PASSIVE IMMUNIZATION BY THE INTRANASAL ROUTE IN EXPERIMENTAL PERTUSSIS

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The mouse has previously been shown to be a suitable animal for the study of both active and passive immunity in experimental pertussis (North, Anderson and Graydon, 1941; North, Keogh, Anderson and Williams, 1939). The method used in previous studies involved the administration of antigen or preformed antibody parenterally followed by infection with living suspensions of Phase I *H. pertussis* by the intranasal route (Burnet and Timmins, 1937). It has since been shown (North and Anderson, 1942) that a far higher grade of active immunity against intranasal infection can be produced in the mouse by administering the antigen intranasally instead of parenterally.

More recently Evans (1944) has reported on the protective action of absorbed pertussis anti-sera given to mice intranasally together with the infecting doses of *H. pertussis*. From the results obtained, Evans concluded that both pertussis anti-bacterial serum and pertussis anti-toxin are able to protect mice against infection by *H. pertussis*.

Experimental immunization by the intranasal route in mice has not been confined to pertussis. There is strong evidence that the administration of influenza immune serum intranasally is more efficient than parenteral injection (Henle, Stokes and Shaw, 1941; Taylor, 1941a; Zellat and Henle, 1941). Krueger and his co-workers (1944) report that atomization of serum is superior to its instillation intranasally, and that mice can be actively immunized by the intranasal route against subsequent intranasal infection.

The present communication records the results of experiments carried out in an endeavour to confirm and extend Evans' observations.

METHODS.

Administration of Sera and Infecting Suspensions.

These were given intranasally by the method of Burnet and Timmins (1937) to mice 18–22 gm. in weight.

In all experiments where serum was administered together with the living *H. pertussis* suspensions, equal volumes of serum (diluted or undiluted) and *H. pertussis* suspensions were mixed immediately before administration. The volume given in all instances was 0·05 ml.

When serum and infecting *H. pertussis* suspensions were administered separately, the volume given of each was 0·05 ml.

Infecting Suspensions of H. Pertussis.

Twenty-four hour growth of Phase I *H. pertussis* was scraped off Bordet-Gengou medium into nutrient broth and standardized against Burroughs Wellcome standard opacity tubes. As a routine in tests where protection against death was the criterion of immunity, the infecting dose was 100×10^6 *H. pertussis*. Allowance was made for the denser suspension necessary when the infecting *H. pertussis* was given together with serum.

Sera Used:

Human.

"Adult contact", "A.C.8". This was a highly potent antibacterial serum used previously as a reference standard (North *et al.*, 1939). It had a low agglutination titre (1:10 with a trace to 1:40) and did not protect mice against the action of pertussis toxin (Anderson and North, 1943).

Individual and pooled children's. These were collected before and after immunization of children by pertussis vaccines. They are described more fully later.

Rabbit.

Anti-toxic. This was prepared by immunizing rabbits with toxin previously treated with 0·2 p.c. formalin (Evans, 1940; Anderson and North, 1943). Its agglutination titre was 1:320.

Anti-bacterial "P 58". This was prepared by immunizing rabbits with formalin-killed Phase I *H. pertussis*. Its agglutination titre was 1:10240.

Mouse convalescent.

Mice were bled 14 days after a sublethal infecting dose of *H. pertussis* given intranasally. The serum contained no demonstrable agglutinins.

Absorption of sera was carried out with living Phase I *H. pertussis* grown on Bordet-Gengou medium for 48 hours. The same absorbing dose (0·015 gm. per ml.) was used for the human serum "A.C.8" and the rabbit anti-toxic serum. Rabbit Serum "P 58" needed a much greater absorbing dose.

Criteria of Immunity.

(a) Reduction in mortality or delay in deaths of mice intranasally infected with a lethal dose of living *H. pertussis*; (b) prevention or control of lung infection in mice infected intranasally with a smaller (usually non-fatal) dose of living *H. pertussis* (North and Anderson, 1942).

The results of experiments have been evaluated statistically by the table of Lowenthal and Wilson (1940).

RESULTS.

Preliminary experiments amply confirmed Evans' finding that the intranasal administration of pertussis antisera gave a high degree of protection against infection with *H. pertussis* given by the same route. As it seemed possible that this protection was due, in part at least, to the *in vitro* action of the anti-sera on the living *H. pertussis* prior to administration of the mixture, experiments in which the serum was given independently of the infecting dose were carried out. These indicated that little protection against certainly lethal infection resulted if the antisera were given after the mice had been infected.

TABLE 1.

Protective action of Pertussis antisera given in mice intranasally, separately from and together with the infecting dose of H. pertussis.

Serum (diluted 1:2)	Time of giving antiserum in relation to infecting dose.	No. of mice.	Result.	Deaths.	Statistical significance against mice receiving normal rabbit serum.
Rabbit antitoxic	24 hrs. before	10	[1]s s s s s s s s s s	0/10	[3]P<0·005
Normal Rabbit	24 hrs. before	10	[2]+2+2+3+3+3+3+4+4ss	8/10	
Rabbit antitoxic	5 hrs. before	10	+4 s s s s s s s s s	1/10	P<0·005
Normal Rabbit	5 hrs. before	10	+3+3+3+3+3+4+4+5+6+6	10/10	
Rabbit antitoxic	1½ hrs. before	10	+7+7+12 s s s s s s s s	3/10	P>0·005
Normal Rabbit	1½ hrs. before	10	+2+3+3+3+4+4+5+5+6+8	10/10	
Rabbit antitoxic	Simultaneously	10	s s s s s s s s s s	0/10	P<0·005
Human anti-bacterial "A.C.8"	Simultaneously	10	s s s s s s s s s s	0/10	P<0·005
Normal Rabbit	Simultaneously	10	+3+3+3+3+4+4+4+5+5 s	9/10	

Experiment terminated after 14 days. [1] s = survived. [2] + = death and the numeral = the day of death. [3] P = probability factor. Values for P of 0·05 or less are significant. If 0·005 or less they are highly significant.

Table 1 shows, however, that a statistically highly significant protection is afforded if an anti-serum is given as long as 24 hours before infection. The slightly poorer protection (see Table 1) afforded when the anti-serum was given 1½ hours before infection may possibly be ascribed to the double traumata inflicted on the test animals within such a short space of time.

In a further experiment rabbit anti-bacterial serum "P58" diluted 1:6 was administered intranasally to groups of 10 mice, 10, 7, 4 days and 24 hours before infection. A statistically highly significant protection against death was afforded mice given serum 4 days before in-

fection. A small degree of protection was present up to the tenth day after administration of the serum.

Although significant protection was not demonstrated when immune serum was administered subsequent to definite lethal infection (see above), protection was observed when a small infecting dose was used (Table 2).

TABLE 2.

Protective action of anti-bacterial rabbit serum (P58) administered intranasally after infection with H. Pertussis.

Dose of serum, P58, given intranasally.	Time interval between infecting dose and administration of serum.	Infecting dose.	No. of mice.	Result.	Statistical significance of number of infected lungs against untreated mice.
0·008 ml.	1 day	50,000	5	++,0,0,0,0	P<0·05
0·008 ml.	3 days	50,000	5	++++,+,+,0,0	
0·008 ml.	5 days	50,000	5	++++,++++,++++,+++,+++	
Nil (control)	Not given	50,000	5	++++,++++,++++,++++,+	
0·008 ml.	1 day	500,000	5	+7,++++,++++,+++,0	
0·008 ml.	3 days	500,000	5	+6,++++,++++,++++,++++,	
0·008 ml.	5 days	500,000	5	++++,++++,++++,++++,c	
Nil (control)	Not given	500,000	5	++++,++++,++++,++++,++++	

Seven days after infection the survivors were killed and the lungs cultured on Bordet-Gengou medium. ++++ = confluent growth, +++ = semi-confluent growth, ++ = many discrete colonies. + = few discrete colonies (<12). 0 = no growth.

Against an infecting dose of 50,000 *H. pertussis* there was a statistically significant reduction in the number of infected lungs against non-treated controls, when serum was administered the day following infection. Some effect was noted with mice treated 3 days after infection.

With an infecting dose of half a million organisms, no protection was observed. Two of the treated mice died and it seems likely that the administration of the serum hastened their death. This point will be further discussed later.

When decreasing concentrations of rabbit anti-bacterial serum were administered together with the infecting dose, a prozone phenomenon was frequently observed. This was not obvious with the rabbit anti-toxic or human anti-bacterial serum.

TABLE 3.

Protective action of Pertussis anti-bacterial serum when administered intranasally together with or 24 hours before the infecting dose of H. Pertussis.

Final dilution of serum in inoculum of 0·05 ml.	Time of giving anti-serum in relation to infecting dose.	No. of mice.	Result.	Deaths.	Statistical significance against death in controls.
1:6	Simultaneously	10	+2+4+4+10+10 sssss	5/10	P<0·05
1:20	Simultaneously	10	+2+4+4+5+6 sssss	5/10	P<0·05
1:60	Simultaneously	10	sssssssssss	0/10	P<0·005
1:200	Simultaneously	10	+2+2+3+4+4+5+5+8s s	8/10	
1:600	Simultaneously	10	+2+2+3+3+6+7+8+10+12 s	9/10	
1:2,000	Simultaneously	10	+2+3+3+3+4 +4+4+6+6 s	9/10	
Control N.R.S. 1:60	Simultaneously	10	+2+2+2+2+2+2+2+3+3+3	10/10	
Control saline	Simultaneously	10	+2+2+2+2+2+2+2+3+3	10/10	
1:6	24 hrs. before	10	+4 sssssssssss	1/10	P<0·005
1:20	24 hrs. before	10	+11 sssssssssss	1/10	P<0·005
1:60	24 hrs. before	10	+11 sssssssssss	1/10	P<0·005
1:200	24 hrs. before	10	+3+4+4+8+10 s s s s s	5/10	P<0·05
1:600	24 hrs. before	10	+2+2+3+4+5+6+7+7+7	10/10	
1:2,000	24 hrs. before	10	+2+2+2+3+3+7+7+12+13 s	9/10	
Control saline	24 hrs. before	10	+2+2+2+2+2+ 2+2+2+3+5+6	10/10	

N.R.S. = normal rabbit serum. Experiment terminated after 14 days.

Table 3 shows the results of an experiment in which increasing dilutions of rabbit anti-bacterial serum were given, in one group of mice together with the infecting dose, and in the other group 24 hours before the mice were infected intranasally. The prozone effect is clearly seen in the group in which the anti-serum was combined with the infecting suspension. Taking the survival of mice as the criterion of protection, the end point is higher (1:200 as against 1:60) in the mice passively immunized prior to infection. If prolongation of life in treated as compared with control animals is considered, a statistically significant protection is shown in both groups with 1:2,000 serum dilution—a total amount of 1/40,000 ml. of undiluted serum per mouse. To obviate the possibility of the prozone effect causing inconsistent results, the practice has been adopted of administering the serum the day prior to infecting mice in all routine protection tests.

TABLE 4.

Comparison of protective effect of Pertussis anti-serum ("P58") given intravenously or intranasally 24 hours before infection with H. Pertussis.

Serum	Dose of serum[1]	Route	No. of mice.	Result	Deaths.	Statistical significance against death.
R.A.B.S. (P58)	0·008	ml. I.N.[2]	10	+2+7 s s s s s s s s	2/10	
R.A.B.S. (P58)	0·0025	ml. I.N.	10	+3+4+4+6+7 s s s s s	5/10	
R.A.B.S. (P58)	0·0008	ml. I.N.	10	+3+4+5+6 s s s s s s	4/10	Vi.v. 0·033 ml. $P<0·05$
R.N.S.	0·008	ml. I.N.	10	+1+1+2+2+2+2+2+2+3+5	10/10	
R.A.B.S. (P58)	0·033	ml. I.V.[3]	9	+1+2+2+2+2+2+2+2 s	8/9	
R.A.B.S. (P58)	0·008	ml. I.V.	10	+1+1+2+2+2+2+2+2+2+5	10/10	
R.A.B.S. (P58)	0·0025	ml. I.V.	9	+1+2+2+2+2+2+2+2 s	8/9	
R.A.B.S. (P58)	0·0008	ml. I.V.	10	+1+1+1+2+2+2+2+2+3+3	10/10	
R.N.S.	0·008	ml. I.V.	9	+1+1+1+2+2+2+2+2+3	9/9	

Experiment terminated 7 days after mice infected intranasally. [1] Dose of serum expressed as actual amount of undiluted serum administered. [2] I.N. = serum administered intranasally. [3] I.V. = serum administered intravenously. R.A.B.S. = Rabbit, anti-bacterial serum. R.N.S. = Rabbit, normal serum.

Table 4 shows the results of an experiment set up to compare directly the protection afforded by intravenous with that by intranasal administration of anti-serum. The mice were infected with an overwhelmingly lethal dose of *H. pertussis* 24 hours after administration of serum. It will be noticed that the great bulk of the mice, except those that received specific anti-serum intranasally, died within 48 hours. There is a statistically significant difference in the number of deaths of mice that got 0·008 ml. of serum intranasally as compared with those that received 40 times that dose (0·033 ml.) intravenously. In addition, amongst those intranasally protected mice that did die there was a delay in death.

TABLE 5.

Comparison of convalescent mouse serum with rabbit anti-serum administered intranasally.

Serum.	Dilution of serum.	No. of mice.	Result.	Deaths.
Convalescent mouse	1:6	6	+2+2+2+2+3+5	6/6
Convalescent mouse	1:20	6	+2+2+2+2+2+2	6/6
Rabbit "P58"	1:6	6	+3 s s s s s	1/6
Rabbit "P58"	1:20	6	s s s s s s	0/6
Nil—control		10	+1+2+2+2+2 +2+2+3+3+3	10/10

The mice were infected I.N. with *H. pertussis* 24 hours after serum was administered and test was terminated 7 days later.

One small experiment (see Table 5) was carried out to compare the effect of convalescent mouse serum with rabbit anti-serum (P58). The mouse serum was obtained from mice fourteen days after being infected with a sub-lethal dose of Phase I *H. pertussis*. Under the conditions of this experiment, no protective antibodies could be demonstrated in the mouse serum.[1]

Evans (1944), as a result of experiments in mice with absorbed and non-absorbed rabbit anti-toxic and rabbit anti-bacterial sera, concluded that pertussis anti-toxin will protect mice against intranasal infection with *H. pertussis*. Experiments along similar lines carried out

[1]Since this report was submitted for publication, protective antibodies have been demonstrated in the serum of mice 30–50 days after a sublethal infection.

by me showed that after absorption the anti-toxic serum retained some protective power whilst the rabbit anti-bacterial serum did not. This result was in accord with Evans' findings. It was found, however, that the human "adult contact" serum (A.C.8) also protected mice after absorption as well as or better than the absorbed anti-toxin. It is therefore concluded that there is no proof that anti-toxin, *per se*, will protect as the human serum "A.C.8" contained no demonstrable anti-toxin (Anderson and North, 1943).

A practical application of the intranasal technique of passive immunisation in mice is exemplified in Tables 6 and 7. An experimental batch of alum-precipitated Phase I pertussis vaccine (Bell, 1941) consistently failed to give statistically significant protection in mice when tested by a previously described method (North *et. al.*, 1941). Table 6 shows the protocol of one of several such experiments.

TABLE 6.

Vaccine.	Route of injection.	Total dose of vaccine in 10^6 organisms.	No. of mice.	Result.	Deaths.	Statistical significance.
Stock Phase I	[2]I.P.	1,000	21	+2+2+3+3+3 +3+3+4+5+5 s s s s s s s s s s	10/21	V A.P.V. I.P. $P<0{\cdot}005$
Stock Phase I	[3]S.C.	1,000	22	+2+2+2+2+3 +3+3+3+3 +4+4+5 s s s s s s s s s	12/22	V A.P.V. S.C. $P<0{\cdot}005$
[1]A.P.V.	I.P.	1,000	22	+2+2+2+2+2 +2+2+2+2+2+3+3 +3+4+4+4+4 +4+4+5 s s	20/22	V Controls Not significant
[1]A.P.V.	S.C.	1,000	25	+2+2+2+2+2 +2+2+2+2+2 +2+2 +2+3+3+3 +4+4+6 s s s s s	20/25	Not significant
Controls		Nil	25	+1+2+2+2+2 +2+2+2+2+2+2+2 +2+2+2+2+2 +2+2+2+3 +5 s s	22/25	

Mice received vaccine in 5 separate doses 200×10^6 at weekly intervals and were infected intranasally 9 days after last immunizing dose. Experiment terminated after 14 days.
[1] A.P.V. = alum precipitated vaccine. [2] I.P. = intraperitoneal. [3] S.C. = subcutaneous.

TABLE 7.

Serum.	Serum dilution.	No. of mice.	Result.	Deaths.	Statistical significance C/f untreated saline control mice.
Pooled children's before	1:2	10	+2+2+2+2+2+2+2+3+3+5+8	10/10	Nil
[1]A.P.V.	1:6	10	+2+2+2+2+2+2 +2+2+2+2+3	10/10	Nil
Pooled children's after stock	1:2	10	+2+3+3+5 s s s s s s	4/10	$P<0{\cdot}05$
Phase I vaccine	1:6	10	+2+2+3+ 3+4+7 s s s s	6/10	$P<0{\cdot}05$
	1:20	10	+2+2+2+2+2+2+3+3+3+3	10/10	Nil
Pooled children's after A.P.V.	1:2	10	+2+2+2+2+2+3+4+4 s s	8/10	Nil
	1:6	9	+2+2+2+2+2+2+4+8+9	9/9	Nil
	1:20	10	+2+2+2+2+2+2+2+4+13 s	9/10	Nil
Rabbit anti-bacterial (P58)	1:60	9	+2 s s s s s s s s	1/9	
	1:600	9	+3+4+8+9+9+13 s s s	6/9	
Saline controls		10	+2+2+2+2+2+3+3+3+3+3	10/10	

Mice infected with lethal dose of *H. pertussis* 24 hours after I.N. inoculation of 0·05 ml. of serum dilution or saline and experiment terminated 14 days later.
[1] A.P.V. = experimental batch of alum-precipitated vaccine.

As the result was unexpected, a small experiment in children was undertaken. Through the co-operation of Dr. Ingram, of the Victorian Children's Welfare Department, a small number of young children, in whom a previous attack of pertussis could reasonably be excluded, were selected, and a sample of serum was obtained from each child.

Half the children then received 3 injections of stock Phase I vaccine, the total dosage being less than 20×10^9 organisms. The others were given two injections of alum-precipitated vaccine with an interval of one month between injections (Bell, 1941). Three months later, serum was again obtained from those of the immunized children who were still in the Home.

Samples of serum from 8 children before and after immunization (4 children in each group) were available for testing. Each sample was first tested individually for protective antibody by the intranasal technique. None of the individual sera collected prior to immunization, nor those from children after injection of alum-precipitated vaccine, gave a significant amount of protection in mice, but the serum of children inoculated with stock Phase I vaccine did give protection.

Table 7 shows the result of a confirmatory experiment in which equal volumes of the individual sera from the differently immunized groups were pooled and tested for protective antibody. Pooled children's serum before injection of alum-precipitated vaccine and rabbit anti-bacterial serum "P58" were used as controls. It will be seen that protective antibodies were not definitely demonstrated in the pooled serum of the children to whom the experimental vaccine was administered, and that the result confirmed that of the active immunity experiments in mice (Table 6).

DISCUSSION.

Several points of interest emerge from the results obtained. Administered intranasally, small doses of pertussis anti-sera are definitely effective in preventing death in mice when given 24 hours prior to infection by the intranasal route. Some protective antibody is present after 10 days.

Henle, Stokes and Shaw (1941) found that to obtain a similar degree of protection against a constant dose of influenza virus administered 24 hours later, five to ten times more immune serum is necessary by the intravenous than by the intranasal route. Our results (Table 4) indicate that, in experimental pertussis, serum given intranasally is at least over 40 times more effective than when given intravenously. 0·3 ml. of a Phase I rabbit anti-bacterial serum of equal agglutination titre to that used in the present work, when injected intraperitoneally, failed to protect mice against death (North *et al.*, 1939). It was only by using a very small infecting dose of *H. pertussis* that significant protection could be demonstrated against intranasal infection not more than 18 hours later.

A possible reason for the marked superiority of intra-nasal over parenteral administration of pertussis anti-serum is suggested by the work of Jones (1924) and Fox (1936). Jones' experiments with rabbits indicated that the walls of the lower respiratory tract are relatively impermeable to antibodies whether contained in homologous or heterologous serum. Fox found that antibodies given intratracheally to rabbits appear only slowly and in small amounts in the blood, whilst, conversely, they are retained in the lungs for a relatively long period.

If serum antibodies are only slowly or in small amounts absorbed from the epithelial surface, they cannot be expected to be as effective against organisms already invading the tissues as against organisms subsequently introduced. Our experience would support such an interpretation.

Taylor (1941b) found that if fluid (normal saline or 10 p.c. normal serum) were given intranasally to mice 1 to 4 days after a sub-lethal dose of influenza virus, the virus content of the lungs markedly increased and many of the mice subsequently died. The multiplication of virus did not occur if 10 p.c. immune serum were substituted for the saline or 10 p.c. normal serum. The experiment shown in Table 2 suggests that the size of the infecting dose of *H. pertussis* in relation to the amount of anti-serum administered may determine whether the serum will be beneficial or actually harmful.

It is likely that inhalation of immune serum droplets would lead to better

results (Zellat and Henle, 1941; Krueger *et al.*, 1944) as the inherent difficulties of intranasal instillation lead to irregularity in distribution of the serum over the mucosal surface of the respiratory tract.

The high grade of protection against subsequent experimental lung infection given by anti-sera, when administered intranasally to mice, is in keeping with the degree of active immunity obtained by administering pertussis vaccine by this route (North and Anderson, 1942). In mice the route of introduction of antibody or antigen is at least as important as antibody potency or antigenicity respectively, and it is possible that the same principle might be found to apply to children.

The complete failure of serum from mice apparently recovering from pertussis to protect other mice (Table 5) was rather unexpected. Further work on the time taken for mice to develop a general immunity following pertussis infection is being undertaken.

SUMMARY.

A high degree of protection against subsequent lung infection in mice is given by intranasal injection of pertussis anti-sera.

A lesser degree of protection is obtained when the serum is given after intranasal infection with *H. pertussis*.

A prozone phenomenon is noticed when an anti-bacterial serum is given together with the infecting dose of *H. pertussis*.

An absorbed human anti-bacterial serum protects mice better than a similarly absorbed anti-toxic serum.

Certain practical and speculative implications of the findings are discussed.

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THE ENDOGENOUS RESPIRATION OF HOMOGENATES OF OYSTER MUSCLE

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Several investigations have been made of various aspects of the respiration of molluscan types. Most of these papers have been concerned with the oxygen consumption of whole organisms and the effects which factors such as temperature, salt content of the medium, biological condition of the animal, time of the year, etc., have upon the observed oxygen uptake. In addition, Chapheau (1932), has studied by means of the Warburg manometric technique, some of the respiratory processes displayed by several isolated tissues of *Ostrea*.

The Australian rock oyster, *Saxostrea commercialis*, is recognized as a widely-distributed euryhaline organism. In common with other lamellibranchs it is able to remain alive for several days when kept out of water. The physiological phenomena observed during this treatment have been studied in several other lamellibranchs (Collip, 1921; Dotterweich and Elssner, 1935; and Dugal, 1938) and to some extent in *Ostrea* by Kühnelt (1938). It seems that the organisms maintain a greatly reduced metabolism during this period and that the shell, by virtue of its high calcium content, and also various tissues, which are relatively rich in calcium, are called upon to counteract possible harmful effects which could be caused by excess carbon dioxide and lactic acid accumulating in the shell fluid. It is also possible that pyruvic acid should be considered in this connection (Humphrey, 1944) and perhaps other, as yet unidentified acids (Wernstedt, 1944).

Further, during a period out of the water there are probably a number of osmoregulatory changes including both salt and water movements occuring between the tissues of the animal and the sea-water which was trapped in the shell-cavity when the animal last took in water from the environment. As a consequence, there could be alterations in respiratory intensity. The changes in oxygen uptake of gill fragments of molluscs in relation to differing salt concentrations of the shell or mantle fluids, have been considered by Pieh (1936).

These findings all combine to make it important to study the effect of various inorganic salts on the oxygen-uptake of oyster muscle.

METHODS.

Oxygen Uptake.

The determinations of oxygen-uptake were carried out in Warburg manometers with flasks of which the volumes were between 20 and 25 ml. All the flasks were used with 0·3 ml. of 20 p.c. KOH in the centre-well and a "KOH-paper" made of No. 40 Whatman filter-paper as recommended by Dixon (1943). The manometers were shaken at 96 cycles per minute in a constant temperature water-bath at 37° C. The small uptakes observed necessitated a longer experimental period than is usual with other more highly developed organisms and the determinations were therefore made over a two-hour period. Usually 3 ml. of suspension were pipetted into each flask.

Preparation of Homogenates.

The muscle suspensions were prepared by dissecting out the adductor muscles of a few oysters and homogenizing them in an instrument similar to that designed by Potter and Elvehjem (1936). Unless otherwise stated, homogenization was carried out for one and a half

minutes at 37° C. at a concentration of 200 mg. of muscle per ml. of final homogenate, the suspending media employed being kept at 37° C. prior to use.

The homogenizers used consisted of about 3 in. of ¾ in. internal diameter Pyrex glass tubing fused onto 3–4 in. of ¾ in. tubing. The smaller tube was sealed and rounded off at the end and a pestle made of glass rod was ground with turpentine and carborundum to fit closely into the ¾ in. tube. The pestle was driven at 450 r.p.m. through a flexible rubber drive jointed to an electric motor. This technique differs from that usually employed in preparing homogenates in that a relatively low speed pestle is used, and that instead of being cut and ground by teeth on the pestle, the muscle is simply disintegrated between the two ground surfaces. From time to time, depending on the amount of use, new pestles had to be ground in to give efficient maceration.

Determination of pH.

At the conclusion of the experimental period the suspensions were pipetted out from the manometer flasks and the pH determined. A Leeds and Northrup portable glass-electrode pH set was used, employing the small sample holder which requires 2 ml. of fluid.

Inorganic Analyses.

Except for the chloride, all inorganic analyses were made after incineration with HNO_3. Individual muscles weighing from 0·3 to 0·9 gm. were placed in 30 ml. Erlenmeyer flasks with 1 ml. of concentrated HNO_3 and 10 ml. of H_2O. The flasks were warmed gently on an electric hot-plate and after about 30 minutes the contents had become reduced to 2–3 ml. of light brown fluid; 10 ml. of H_2O were added and the contents evaporated to about 5 ml. The fluid in each flask was then diluted to 20 ml. With each batch of analyses, standard solutions of the ions being examined were subjected to this ashing procedure.

Potassium. 1 ml. of the solution was neutralized with NaOH to phenolphthalein and diluted to 2 ml. This was then analysed according to the procedure of Kramer and Tisdall (1921). Following the recommendation of Horton (1930), the factor required to convert the amount of permanganate used into the potassium present, was derived from a series of analyses of standard solutions. The factor found was 1 ml. of N/50 $KMnO_4 \equiv 0{\cdot}140$ mg. K.

Sodium. 1 ml. of the solution was neutralized with ammonia to phenolphthalein and diluted to 2 ml. This was then analysed by the method of McCance and Shipp (1931).

Calcium. 5 ml. of the solution were analysed by the method of McCance and Shipp (1933).

Magnesium. 4 ml. of the supernatant fluid from the calcium oxalate precipitation were treated according to the method of McCance and Shipp (1933). After drying, the $MgNH_4PO_4$ precipitates were dissolved in 0·4 ml. of 5N sulphuric acid and the phosphorus estimated by the method of Fiske and Subbarrow (1925).

Chloride. Individual muscles were analyzed by the method given in the A.O.A.C. Handbook (1940). 5 ml. of N/10 $AgNO_3$ and 5 ml. of concentrated HNO_3 were added to each muscle.

Materials.

The oysters used were 2–3 year old stock of *Saxostrea commercialis* from the C.S.I.R. Experimental Farm at Georges River, New South Wales.

All the chemicals used were of "Analar" grade.

RESULTS.

Inorganic Composition of the Muscle.

In Table 1 are given the results of the analyses of oysters taken from sea-water of which the salinity has fluctuated very slightly around $35^0/_{00}$ over several years. The muscles were dissected out as soon as possible after the animals were removed from the water; this period would be about 5 hours. In calculating the variance, 15 degrees of freedom were used and the standard error of the means was obtained from the usual formula S.E. $\pm \sigma/\sqrt{N}$.

Conditions for Homogenization.

The largest amount of muscle which was homogenized at the one time was about 4 gm. This required a minimum of one minute's grinding to give uniform disintegration. The results set out in Table 2 do not show a pronounced temperature effect. Only at 37° C. is there a definite change of activity with duration of homogenization. As a routine, one and a half minutes at 37° C. was used.

TABLE 1.

Composition of muscle.

In each case the figures represent mg./100 gm. of muscle. The weights given as mg. are those of the whole muscle.

Weight	K	Na	Ca	Mg	Weight	Cl
715	472	354	51·6	72·0	437	669
533	553	353	39·0	76·5	947	787
586	480	329	51·2	72·0	509	764
452	385	364	75·9	75·1	603	732
499	422	314	64·1	71·9	470	827
513	338	317	25·0	77·5	438	685
553	319	380	30·3	85·3	648	852
287	342	495	58 7	105·0	682	852
483	427	406	51·1	85·1	543	718
449	415	350	41·1	73·0	540	692
684	295	372	51·5	71·4	957	690
575	370	335	41·9	67·9	520	628
598	288	482	41·6	110·0	452	735
358	336	350	35·9	79·0	276	712
570	370	402	39·5	85·8	387	641
711	295	363	33·8	71·3	467	751
Mean						
535·4	381·7	372·9	45·76	79·93	554·8	733·4
Standard error						
29·4	18·9	13·0	3·28	3·03	45·9	17·3

TABLE 2.

Effect of temperature and duration of homogenization.

The muscle was homogenized as 200 mg./ml. in water and 3 ml. of the suspension was placed in each flask with no further additions. The temperature was controlled during homogenization by ice- or water-baths. Figures are μl O_2/2 hrs.

	2° C.	20° C.	37° C.
1 minute	30·8	34·0	31·8
3 minute	32·6	29·0	25·6
5 minute	30·1	32·0	24·6

Concentration of Suspension.

This was considered in two ways. Firstly, the effect of dilution on a relatively concentrated homogenate and secondly as the effect of homogenizing at various concentrations. Diluting the suspension from 400 to 50 mg./ml. increases the O_2-uptake/100 mg. whilst homoganizing at a concentration of 100 mg./ml. seems to give the greatest uptake per 100 mg.

TABLE 3.

Concentration of suspension.

Figures are μl O_2/2 hrs. 3 ml. of fluid per flask; no additions.

Concentration mg./ml.	Homogenized at 400 mg./ml. and then diluted.	Homogenized at the concentration given.
400	3·7	4·8
300	5·1	6·3
200	5·3	6·3
100	5·9	8·9
50	7·8	7·8

Effect of pH.

This was investigated in relation to the various suspension media employed. In most of the experiments the pH was regulated with one-tenth of a volume of M/5 Sörensen buffer; in the others the pH was brought to the desired value with N/10 HCl or NaOH. In all cases

the pH was determined at the end of the two hours. In some cases the pH was taken before and after the experiment; there was never a change of more than 0·1 (usually a decrease). The curves obtained are shown in Fig. 1.

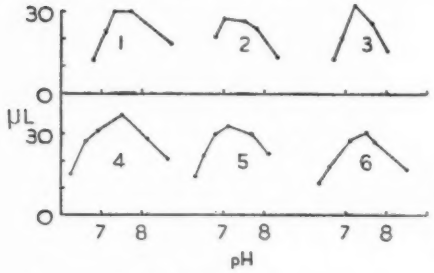

Fig. 1. Effect of pH on oxygen uptake. Figures are as μl O_2/2 hrs. 600 mg. of muscle in each flask. All have phosphate buffer except 2. 1. Water. 2. Water (no buffer). 3. "Oyster solution". 4. Ringer solution. 5. Sea-water. 6. Krebs-Henseleit solution.

Composition of Medium.

Several different inorganic solutions were used as suspension media. A Ringer solution and a modified Krebs-Henseleit (1932) solution with the compositions given below were used as representative of media isotonic with mammalian serum.

gm./100 ml.	Ringer	Krebs-Henseleit
NaCl	0·864	0·900
KCl	0·023	0·046
$MgSO_4 \cdot 7H_2O$	0·038	0·038
$CaCl_2$	—	0·037

In an attempt to increase the amount of cytolysis (Elliott and Libet, 1942) water was also tried. Also, since under normal conditions the muscle is exposed to sea-water, an artificial sea-water of the following composition was prepared. (Lyman and Fleming, 1940).

	gm./100 ml.
NaCl	2·348
Na_2SO_4	0·392
KCl	0·066
KBr	0·010
$MgCl_2$	0·498
$CaCl_2$	0·110

Finally, following on the values arrived at for the inorganic composition of oyster muscle, a solution was prepared as below. This contained the various ions in approximately the same concentrations as found in the intact muscle. This medium will be referred to as "oyster solution".

	gm./100 ml.
NaCl	0·949
KCl	0·730
$MgSO_4 \cdot 7H_2O$	0·797
$CaCl_2$	0·124

As can be seen from Fig. 1, alterations in the composition of the medium did not affect the respiration very much

With reference to the euryhalinity of the organism, O_2-uptakes were determined in the presence of various dilutions of the artificial sea-water. The results were very variable and there was no definite trend from one dilution to another (Table 4).

TABLE 4.

Various dilutions of sea-water.

Homogenized at 500 mg./ml. in water; 1 ml. was placed in the cups which contained 150 p.c. sea-water. pH's were from 7·2–7·4; in only one series was M/50 buffer present.

Final concentration of sea-water.	Buffer.	No buffer.
100 p.c.	13·7	22·8
75	15·3	24·3
50	14·5	21·2
25	25·1	22·7
10	24·5	28·4
0	19·2	22·8

The effect of the addition of one salt at a time was considered as follows. The muscle was homogenized at 300 mg./ml. in water and 2 ml. aliquots added to concentrated salt solutions. No buffers were used, but the pH was adjusted to be constant to 0·1 within a series. The figures (Table 5) show no regular effect with alterations in the salt concentration. Ca and Mg. seem to inhibit slightly while Na and K accelerate the O_2-uptake.

TABLE 5.

Variation due to different dilutions of salts.

Salt concentration.	NaCl	KCl	$MgSO_4$	$CaCl_2$
0·38 M.	21·1	14·2	16·1	10·7
0·23	20·3	16·8	19·8	10·0
0·15	20·2	17·1	21·6	9·1
0·09	23·0	13·8	18·8	11·8
0·05	19·6	20·6	16·0	13·4
0·02	23·6	20·6	22·4	15·0
—	18·2	13·7	26·4	18·7

The results in Table 6 confirm the accelerating effect of Na and K and the inhibiting effect of Mg and Ca.

TABLE 6.

Equal concentrations of various salts.

Conditions as for Table 5. Salts were present at a final concentration of 0·15 M.

	μl. O_2/2 hrs.
Water	27·9
NaCl	36·7
KCl	32·4
$MgSO_4$	19·5
$CaCl_2$	17·5

TABLE 7.

Inorganic composition of tissues.

All the values are as mg. p.c.

	French oyster.	Shellfish.	*O. edulis.*	*S. commercialis.*	Frog.
K	180	345	258	382	320
Na	609	99	505	373	50
Mg	—	17	42	80	27
Ca	—	22	186	46	24
Cl	910	241	815	733	53

DISCUSSION.

Composition of the Muscle.

Table 7 sets out for comparison some figures relating to the inorganic composition of other molluscs. Katz (1896) analyzed shellfish muscle and McCance and Shipp (1933) the whole tissues of *Ostrea edulis*; also, Krogh (1938) determined the Cl, Na and K in the muscles of French oysters (no species stated). For comparison some figures for frog-muscle have been recalculated from Fenn (1936).

The higher content of Na, Cl and Ca in *Saxostrea* as compared with frog muscle is readily explainable from its marine environment and the proximity of the muscle to the shell. The reason for the high Mg value is not so clear, especially in comparison with the figures of Katz. However, it is a well known fact that a number of marine invertebrates possess the power of concentrating in their tissues elements which occur only in traces in sea-water. The results given by Krogh were obtained on an acid extract of the muscle; this makes them not directly comparable with the other figures. For the Cl content of the muscle of *Mytilus californianus* Fox (1941) gives a mean value of 720 mg. p.c., which is close to the 733 obtained for *Saxostrea*.

Krogh (1939) cites *Ostrea* as a typical marine invertebrate in osmotic equilibrium with its environment over the range through which it is euryhaline. He points out that in the molluscs so far examined, the ionic concentrations in the tissues are often much lower than those in the blood. This is substantiated by a comparison of the figures given for *Saxostrea* and those published by Kumano

(1929), for the blood of *O. circumpicta* which has an inorganic composition little different from that of the sea-water in which it is normally found. Kühnelt (1938) found that the Ca content of the shell fluid of *O. edulis* could change from 30 to 50 mg. p.c. when the animals were left out of the water for six days. Similarly Krogh (1938) has shown that the blood and tissues of the oyster can come into equilibrium with dilute sea-water after one to two day's immersion. From this it would appear that the muscle can function in a fairly wide range of total salt concentration and also of an individual ion such as Ca.

Effect of pH on Respiration.

The results presented in the various graphs show pH-optima which vary around 7·4. This is in accord with the findings of other workers on mammalian tissues. Kreps (1929a), investigating the respiratory exchange of *Balanus crenatus* found no change when the pH was varied between 7·2 and 8·2; he was working with whole organisms placed in sea-water of differing pH and he points out that although the external pH varied, that of the internal fluids was probably kept constant by virtue of the buffering that can be brought about by the shell. Kokubo (1929), reports that the normal blood pH of *O. circumpicta* is from 7·15 to 7·68 depending on the season, and that when CO_2 accumulates in a period out of the water, the pH can fall to low values. Contrary to Kreps, he states that changing the pH in the external medium alters the blood and tissue pH. Moreover, he claims that the blood pH can be varied from 5·40 to 8·45 without death. However, he gives no indication that any experiments were conducted for periods longer than forty-eight hours; he states that normal physiological reactions were given more frequently by the animals at high rather than at low pH values. This, and the findings of Kreps may be related to the more gradual slope of the curves in Fig. 1 towards the higher pH's.

Effect of salts.

Kreps (1929b), investigated the effects of different external salinities on *Balanus* respiration. Placing the whole animal in water, he found that the respiratory exchange was unaffected by salinities between 12 and $35^0/_{00}$. There was less respiratory activity below $10^0/_{00}$. Also Borsuk and Kreps (1929), exploiting the poikilosmotic properties of *Balanus* changed the salt content of the "*milieu intérieur*" and determined the O_2-uptake in air. Up to $6^0/_{00}$ low values were observed, from 6 to $10^0/_{00}$ there was a sharp increase and from then on, only a slight rise to $35^0/_{00}$. Table 4 shows no such definite trend for *Saxostrea* but might be interpreted as indicating highest uptakes in dilute sea-water (from 4 to $9^0/_{00}$).

Pieh (1936), showed that for some homoiosmotic types, replacing the normal medium (32 or $15^0/_{00}$ sea water) by equivalent NaCl solutions increased the O_2-uptake of isolated gills by up to 75 p.c. The permeability of the gills under these conditions was lowered, more water being taken up in NaCl than in sea-water. A. E. Hopkins (1932) showed that the sensory stimulation of *O. virginica* was greatest with KCl, followed by NaCl and finally by $MgCl_2$. With regard to the figures in Table 5, there seems to be no definite change in respiratory intensity with change in NaCl concentration. From 0·02 to 0·05 M is the optimum for KCl, while $MgSO_4$ and $CaCl_2$ inhibit respiration. Krebs and Eggleston (1938) point out that Ca inhibits oxidations in minced tissues. From the relatively high amount of Ca in oyster muscle and also the close relation between the muscle and the shell-fluid which at times dissolves Ca off the shell, it might have been expected that Ca would not have an adverse effect upon the O_2-uptake. However, such is not the case. Also, as Table 6 shows, at a concentration of 0·15 M, NaCl and KCl stimulate respiration, whereas $MgSO_4$ and $CaCl_2$ inhibit.

From the above results it appears that *Saxostrea*, as would be expected from its position as a relatively euryhaline, poikilosmotic marine invertebrate, is an organism with muscular tissue of a low respiratory intensity. Also, the intensity of respiration is not greatly affected by salt concentration. The pH effects are

fairly well-marked and the pH-optima are close to the normal blood pH of related species.

Assuming that the amount of water in the muscle is 80 p.c. (Humphrey, 1941), the highest Q (μl O_2/hr./mg. dry weight at 37° C. in air) obtained was —0·21. This is of the same order as that observed by Chapheau (1932); he gives no indication as to how the estimation was done other than saying that the Warburg technique was used. His results showed that for 3-year-old oysters the Q was —0·25 and for 1¼-year-old stock, —0·46. H. S. Hopkins (1930) working on blocks of muscle which were not suspended in any fluid found Q's of from —0·25 to —0·40 for *O. virginica*. Also, Galtsoff and Whipple (1930), using the Winkler technique on whole oysters, obtained Q's of from —0·65 to —1·50.

SUMMARY.

The mean values, in mg. p.c., for the concentration of some elements in the adductor muscle of *Saxostrea commercialis*, were found to be K, 381·7 ± 18·9; Na, 372·9 ± 13·0; Ca, 45·76 ± 3·28; Mg., 79·93 ± 3·03; Cl, 733·4 ± 17·3.

Muscle could be homogenized at temperatures of from 2 to 37° C. for periods varying from 1 to 3 minutes with little change in respiratory intensity.

The greatest O_2-uptake/100 mg. of muscle was observed at a concentration of 100 mg. muscle/ml. of homogenate.

Physiological media such as those of Ringer and Krebs-Henseleit, various dilutions of artificial sea-water, and a special "oyster solution" gave only small variations in O_2-uptake.

Na and K accelerated, but Ca and Mg. inhibited, respiration.

The Q_{O_2} of the homogenates was —0·21.

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A MODIFIED SALK TEST FOR *IN VITRO* TITRATION OF INFLUENZA ANTIBODIES

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For any studies of the distribution of antibody against influenza A and B in a large series of children's sera, it would be obviously desirable to have an *in vitro* method which would distinguish clearly between sera with no antibody and those with any amount of antibody, provided we could be certain that this was of specific origin.

The general experience is that with human serum, mouse neutralization tests are the most reliable index of small amounts of antibody. With an active test virus it is possible to detect about 0·1 p.c. of the amount of antibody present in a potent post-infective serum. The method of antibody titration *in ovo* first described by Hirst is almost equally sensitive, the smallest detectable amount of antibody being of the order of 0·1 p.c. of the largest amounts found in immune sera (Burnet, Stone and Kerr, 1946). Using haemagglutination methods, small amounts of antibody are not normally detectable owing to interference by nonspecific inhibition at higher serum concentrations. This nonspecific inhibition is more potent in ferret and rabbit sera than in human, but even with human sera it is more than sufficient to make ordinary titrations unreliable for low amounts of apparent antibody, particularly with some virus strains.

The method to be described utilizes the fact that the nonspecific inhibitory titre is greatly influenced by fine differences amongst the red cells used in the haemagglutination test. If the cells used are taken only from fowls specially selected from this point of view, much more satisfactory results can be obtained.

EXPERIMENTAL.

General Technique.

All tests were made by observation of the pattern in which cells are deposited at the bottom of test tubes 10 mm. in diameter, from a total volume of 0·75 ml. of saline. Except for special reasons, the red cells were used at 0·5 p.c. concentration, giving a final concentration in the standard three-volume set up of 0·167 p.c.

In the preliminary work two sera were chiefly used, one EDW obtained *post mortem* from a child of two years and showing no evidence of antibody when titrated *in ovo*, the other ROS II a high titre human serum, obtained after an attack of influenza B.

Since in this work we were chiefly interested in influenza B antibodies, most of the work was done with the current influenza B strain, MIL-B, isolated in October, 1945 (Burnet, Stone and Anderson, 1946).

Differences Between the Cells from Different Fowls.

Blood samples were taken in citrate solution from the wing vein of 23 adult white leghorn fowls. The cells were washed twice, and made up in the first instance to 2 p.c. suspension in saline. At this strength several were used to titrate a stock allantoic virus of strain MIL-B, and a standard virus dilution, corresponding to one agglutinating dose with 2 p.c. cells of the most susceptible type, was prepared. Using this standard dilution of virus, the two sera, EDW and ROS II, were titrated, using cells from each of the 23 fowls. The results are shown graphically in Fig. 1.

[1] This work was carried out under a grant from the National Health and Medical Research Council.

The points of chief interest are (1) the wide range of titres shown with the serum containing no antibody and (2) the more uniform results obtained in the titration of a high titre immune serum. The third point to be noted is that the cells which give the highest titre in straight virus titrations are those which allow the lowest nonspecific inhibitory titre to be shown by a serum containing no antibody. Finally, it will be observed that only 4 of 23 fowls give cells which are satisfactory in the sense of allowing titres of less than 10 to be obtained with normal serum.

Failure to Obtain Negative Results from Normal Sera with Certain Virus Strains.

The results obtained with the strain MIL-B could be duplicated more or less closely with recently isolated A strains, inasmuch as the same gradation in the nonspecific inhibitory titre given by a non-immune serum was noted from one sample of red cells to the next. However, those strains derived from sporadic infections in 1945–46 gave a higher nonspecific inhibitory titre than MIL-B with similar cells. In other words, the range of nonspecific inhibitory titres, which in the case of MIL-B reached 1:5 in the four lowest values, was set higher in the case of the 1945–46 strains, of which MIL-A is an example.

The strain Melb. M has been passaged many times since isolation in 1935. The nonspecific inhibitory titre of the non-immune serum Wad against Melb. M was found to be $<1:6$ with all types of fowl cell used.

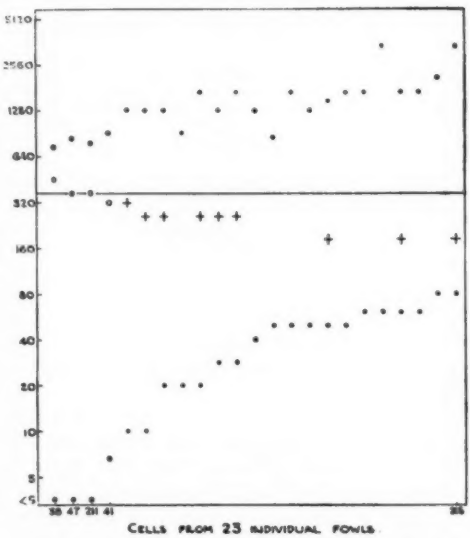

Fig. 1. Titres obtained with cells from 23 different fowls. Each vertical series of points corresponds to cells from one fowl. From above downward they are (1) antihaemagglutinin titre of an active immune serum ROS II. (2) Direct haemagglutinin titre of the stock virus MIL-B used in all tests. (3) Apparent antihaemagglutinin titre of a serum containing no antibody.

The points corresponding to "satisfactory" cells are shown with open circles.

TABLE 1.

Titration of non-immune serum Wad using various samples of fowl cells.

Fowl.	Mil-A	Melb. M
211	9	<6
47	10	<6
83	15	<6
78	36	<6
61	65	<6

Cells of fowl 211 and 47 are of full susceptibility. See Fig. 1.

Table 1 gives some representative results. It will be seen, therefore, that in the type of investigation we are contemplating (a survey of children to determine at what ages influenza A and B are first encountered) in addition to using suitable red cells it is also necessary to choose suitable strains of virus, which show low nonspecific inhibitory titres. This requirement may at times clash with the other requirement, that the strains should be of the antigenic type characteristic of recently current influenza in the community.

A finding which may allow the partial differentiation of nonspecific inhibition from true specific inhibition is that the endpoint of a weak or strong specific neutralization usually moves outward if the titration tubes, after being read, are reshaken and again allowed to settle. A nonspecific inhibition, on the contrary, remains at approximately the same titre. The evidence

on which this statement is based is given in Table 2, which shows the degree of correlation between the *in vitro* results (on the above criterion) and *in vivo* tests for small amounts of antibody.

The sera included in this Table were all obtained from children less than 14 years of age; after inactivation at 56° C. for 30 minutes, merthiolate 1:10,000 was added, and the sera stored at 4° C. in tightly corked tubes.

In vivo tests were performed, using the technique of inoculation of the allantoic cavity of 11-day-old chick embryos. The seed virus used was amniotic fluid of high titre, harvested from 18-day-old embryos; glycerol was added to 30 p.c. concentration, and this stock maintained at —10° C. in corked tubes. It was found that under these conditions, the titre was maintained over the period of the tests *in ovo*.

A titration was made in eggs of the seed virus, and the dilution determined, 0·05 ml. of which would regularly initiate infection in approximately 50 p.c. of embryos. This dilution of virus was used in the control eggs. A suspension of seed virus was diluted to give 20 times this concentration, and equal volumes of this dilution and undiluted serum were mixed and held at 37° C. for 30 minutes. The mixture was then chilled, and 0·05 ml. was inoculated into the allantoic cavity of 11-day-old chick embryos, eight eggs being employed for each serum and a similar number for the control virus dilution.

With the virus strains used, the full titre was shown by the third day in the absence of serum; and as a routine, samples of allantoic fluid were taken and tested for haemagglutinin at this time. Samples from the test eggs were also taken at three days, but negatively reacting eggs were replaced in the incubator and tested again at four and at five days. It was considered that antibody was present in a serum if virus was detected at five days in less than half the living embryos which had received the virus-serum mixture.

The *in vitro* tests for anti-haemagglutinin activity were made, using virus in the form of infected allantoic fluid of the required strain. A stock of fluid, containing 10 p.c. of glycerol, was maintained at ± 4° C. and used for all the tests reported.

The figures recorded in the Table as haemagglutinin inhibitory titres, denote the dilution of serum in the presence of which "+" (partial) agglutination of 0·5 p.c. fowl red cells was produced by five agglutinating doses of virus. The first reading was made after the tubes had stood for one hour at 20° C., and the racks were then reshaken. This was repeated at intervals of 45 minutes until three or four readings had been made. The red cells used were from fowls known to provide cells which gave the lowest nonspecific inhibitory titre with the corresponding virus.

If we adopt the results of *in ovo* neutralization experiments as the criterion, we can divide the sera into two main groups with a small number of intermediates. The first group shows no evidence of antibody, while sera of the second group contain sufficient antibody to prevent infection in all eggs inoculated with ± 10 infective doses of virus.

When the results of haemagglutinin titrations, made as described above, are compared with the *in ovo* tests (Table 2), it is found that with sera of the first group (containing no antibody) the figures for haemagglutinin inhibitory titre obtained at successive readings remain practically constant.

Since those of our sera which contained specific antibody were obtained from children whose most recent influenzal infection might well have occurred years before, the amount of antibody present was very small. So, with sera of the second group, the first readings are of approximately the same magnitude as those of the first group, but characteristically show a twofold or greater rise in titre over three or four successive readings.

From results of this sort, we have adopted the empirical rule that inhibition of agglutination of the type represented by Cas (9-9-10), Hin (6-6-6), is due to a nonspecific factor incapable of influencing growth of virus *in ovo*, and is not evidence that the subject has been infected with influenza virus.

As in every serological survey of comparable character, a few sera are found which can be classed as positive or negative only by completely arbitrary criteria; examples in the Table are Duc, Har, Bus and McEv.

The figures recorded for the nonspecific inhibition of Melb. M are all less than six. The corresponding figures for seven of these sera were determined against MIL A, using cells of fowl 40, which gives a low nonspecific inhibitory titre.

Table 3 shows that despite the much higher apparent titre, this does not change on successive shaking and resettling.

Modified Salk Technique for* in vitro *Antibody Titration.

As a result of this work we have adopted the following technique for antibody titrations. Except in minor details, it corresponds very closely with Salk's technique, and should be regarded only as a modification thereof.

Reagents.

Red Cells. These are obtained from fowls previously selected as providing highly susceptible cells. With continued bleeding, at least one fowl has shown a change toward less satis-

TABLE 2.

Comparison of in ovo *and* in vitro *titrations of serum influenzal antibody titres.*

(1) Virus B Mil-B serum.	Proportion of living eggs containing virus.	Haemagglutinin inhibitory titres.			
Cas	7/8	9	9	10	
Mal	3/3	<6	<6	<6	<6
Pro	3/4	<6	<6	<6	
Hin	3/3	6	6	6	
O'Far	4/5	6	<6	<6	
Bus	5/7	9	10	12	
Mat	4/7	6	5	6	6
Gat	0/7	6	9	20	28
Pow	0/9	640	1280		
Rob	0/8	<6	7	9	12
Has	0/6	9	12	18	
Sca	0/6	6	9	18	
Kni	0/7	6	12	18	24
Joh	0/5	6	9	12	20
Tin	0/4	9	24	30	48
Con	0/6	12	36	48	72
Duc	1/7	<6	6	9	9
Har	2/5	6	9	10	12
Virus A. Melb. M.					
Bat	6/6	<6	<6	<6	<6
Cab	7/8	<6	<6	<6	<6
Wad	6/6	<6	<6	<6	<6
Bar	3/4	<6	<6	<6	<6
Moo	6/6	<6	<6	<6	<6
Smo	6/7	<6	<6	<6	<6
Pit	5/7	<6	<6	<6	<6
New	8/8	<6	<6	<6	<6
Pro	6/6	<6	<6	<6	<6
Fie	0/7	12	18	32	
Sca	0/6	<6	7	12	
Smi	0/7	9	24	36	
Can	0/3	<6	8	10	
D'ar	0/5	9	12	18	
McEv	1/5	9	9	12	

TABLE 3.

Haemagglutinin inhibitory titres of non-immune sera against MIL-A virus.

Serum.	Titre.			
Bat	18	16	16	18
Wad	24	36	24	36
Bar	72	80	80	80
Moo	72	72	72	90
Smo	36	36	48	48
New	36	40	30	36
Pro	24	18	24	24

factory cells, so that it is advisable to check each batch of cells by including a titration of a known negative serum.

In view of the results obtained by Miller and Stanley (1944), it is desirable that the cells should be used within 48 hours of being obtained, although we have not found any significant change in nonspecific inhibitory titres in a few comparative tests of three and four-day-old cells against fresh cells. Our routine is to collect in citrate solution, wash once with normal saline and once with calcium saline before suspending in calcium saline as a 2 p.c. suspension of packed cells. This is diluted 1:4 when 0·5 p.c. cells are required.

Virus. A strain giving reasonably high titre allantoic fluids (end point 160), and capable of showing low nonspecific inhibitory titres is needed. When a long series of neutralization

tests with the same virus is needed; we have prepared a stock of allantoic fluid virus and added 10 p.c. by volume of sterile glycerol. This inhibits urate precipitation, and we have found the titre to be well maintained in the refrigerator.

The standard dilution of virus to be used is that which, in a three-volume system with 2 p.c. cells (final concentration 0·67 p.c.), gives at 20° C. our one-plus degree of partial agglutination. This point can be estimated to almost 10 p.c. accuracy by using a pipette delivering 40 drops per ml., and preparing 0·5 ml. mixtures of virus and saline over the range from 5 to 15 drops of approximately correct virus strength. With one drop difference from tube to tube, it is nearly always possible to say that one tube corresponds more closely to our conventional standard than any other. The standard was chosen in the first place as the pattern which was changed most markedly by a small change in concentration of virus in either direction.

When this value is obtained, the required amount of virus dilution in calcium saline is prepared, kept cold, and used the same day.

Serum. All serum samples are inactivated at 56° C. for 30 minutes, and are stored in the cold in tightly corked tubes with 1:10,000 merthiolate.

The Test.

Serum is diluted in 0·25 ml. volumes from 1:5 or 1:10 usually for six doubling dilutions; 0·25 ml. of virus, diluted to correspond to one agglutinating dose with 2 p.c. cells, and 0·25 ml. of 0·5 p.c. fowl cells from a suitable bird, are added. The racks are shaken, left an hour at room temperature, and read. They are reshaken, and read again when negative tubes have settled to a compact button of cells, i.e. in 70–90 minutes. The final reading is the one used. The degree of change between first and second readings will help in allowing a decision as to whether a low apparent titre is to be regarded as specific or nonspecific.

DISCUSSION.

The usefulness or otherwise of the method for serum titration, that we have described, must be determined on an empirical basis, by its use in the problem for which it was designed, namely, the collection of serological evidence in regard to the epidemiology of influenza A and B in young children. This work is in active progress.

The influence of the fowl red cells, in modifying the apparent titre of sera tested by one or other modification of Hirst's method, has been noted by Hirst (1943), and by Miller and Stanley (1944); but no detailed study of the effect has been published, and American workers have usually overcome the difficulty by using large pools of fowl cells from many different birds.

It has seemed worth while to attempt a simple theoretical treatment of the problem presented by the use of fowl cells of differing susceptibility as indicators of the endpoint of a virus antibody titration. The treatment is based on simple mass action principles, and at best can be only a first degree approximation.

A. The agglutination of erythrocytes by virus involves the reversible reaction Virus + cells $\rightleftharpoons$ Virus-cell combination — (1). Two factors may affect the concentration of virus required to cause standard "+" agglutination of different fowl erythrocytes:

(a) The number of virus particles adherent to each erythrocyte at the "+" point may vary with the type of fowl-cell used.

(b) The same number of adsorbed virus particles per cell is needed to produce standard agglutination, but the cells may vary in their adsorptive "affinity" for virus, so that for cells which give relatively low titres, a greater concentration of free virus must be present in the medium to give the necessary concentration of effectively adsorbed virus.

We assume the second factor is of primary importance.

B. Inhibition by serum may be due to the presence and activity of specific immune antibody, or to a nonspecific inhibiting effect of normal globulin. In ordinary circumstances, we may assume that in the two reversible systems: Antibody + virus $\rightleftharpoons$ Inactive combination — (2); Normal globulin + virus $\rightleftharpoons$ Inactive

combination — (3), a much higher globulin concentration will be necessary to produce a given reduction in free virus, than will be needed of specific antibody

The further discussion will be concerned only with conditions at equilibrium, in the mixtures giving standard "+" agglutination. We have assumed that a constant amount, "a", of virus is adherent to the cells, irrespective of their type, whether "good" cells (susceptible), or "poor" cells giving high nonspecific inhibitory titres. There will also be present in the free state, a concentration of virus which can be taken as "b" for good cells, and $b+\delta$ for poor cells. Then $a+b=1$ agglutinating dose (A.D.) for good cells, and $a+b+\delta=1$ A.D. for poor cells. If the total amount of virus in the system is maintained constant at n A.D. as measured with good cells, this amount will be in all cases $n\ (a+b)$ units.

If $[A]$ represents the concentration of free antibody or globulin, $[V]$ that of free virus, and $[AV]$ that of antibody or globulin combined with virus, then equilibrium in reactions (2) or (3) can be represented $\frac{[AV]}{[A][V]} = \frac{1}{K}$ or $[A] = [AV].\frac{K}{[V]}$.

In the presence of red cells showing standard agglutination $[V]=b+\delta$ (δ being zero if fully susceptible cells are used), and the amount of virus combined with inhibitor (antibody or globulin) is equivalent to $[AV]$, and $=n(a+b)-(a+b+\delta)$

$$=(n-1)\ (a+b)-\delta$$

The ratio R between the amount of antibody necessary when "poor" cells are used, *i.e.* $[A]_p+[AV]_p$ and when "good" cells are used, *i.e.* $[A]_g+[AV]_g$ will be

$$\frac{[AV]_p \frac{K}{[V]_p} + [AV]_p}{[AV]_g \frac{K}{[V]_g} + [AV]_g}$$

$$= \frac{[AV]_p}{[AV]_g} \cdot \frac{\left(\frac{K}{b+\delta} + 1\right)}{\left(\frac{K}{b} + 1\right)}$$

Since the amount of combined antibody is taken as equivalent to the amount of virus combined with it, this ratio becomes

$$\frac{(n-1)\ (a+b)-\delta}{(n-1)(a+b)} \cdot \frac{1+K/(b+\delta)}{1+K/b}$$

$$i.e.\ R = \left\{1 - \frac{\delta}{(n-1)(a+b)}\right\} \cdot \frac{1+\frac{K}{b+\delta}}{1+K/b}$$

If instead of adding to the system a constant amount of virus, $n(a+b)$, we add n agglutinating doses for the particular cells being used [i.e. $n(a+b+\delta)$ for poor cells, $n(a+b)$ for fully susceptible cells], a similar chain of reasoning leads to the expression:

$$R_1 = \left(1 + \frac{\delta}{(a+b)}\right) \cdot \frac{1+K/(b+\delta)}{1+K/b}.$$

which is independent of the number of agglutinating doses of virus employed.

When K is small, with highly active antibody, R_1 in the limit becomes $1 + \frac{\delta}{(a+b)}$, which is the ratio between the amounts of virus required to give standard agglutination in the absence of serum, and $R=1-\frac{\delta}{(n-1)(a+b)}$.

In the results shown in Fig. 1, the ratio $\frac{a+b+\delta}{a+b}$ for good cells 211 and poor cells 215 is $\frac{2}{1}$ *i.e.*, $\delta = (a+b)$.

From the above expression $R = 1 - \left(\frac{1}{n-1}\right)$ under these conditions; i.e. except with a very small test dose of virus, the difference in titre would not be detectable by the rough methods used, if the antibody-virus union could be regarded as a practically irreversible one. On the other hand, $R_1 = 1 + 1 = 2$; i.e. if we double

the amount of virus to allow for the lessened sensitivity of the indicator cells, the apparent serum titre with the poor cells will be half that with good cells.

With larger values of K (nonspecific inhibitor) R becomes in the limit $\left\{1-\frac{\delta}{(n-1)\,(a+b)}\right\}\cdot\frac{b}{b+\delta}$ and if n is large the value is determined essentially by $\frac{b}{b+\delta}$. The smaller this value, the smaller will be the amount of serum required to "neutralize" a standard dose of virus.

The finding, that on repeated shaking and resettling the apparent inhibitory titre increases with true antibody but remains approximately constant with nonspecific inhibitor, is in line with general immunological experience. With the very dilute systems being considered, the concentration of antibody needed to come to final equilibrium with the constant amount of virus and red cells will be very low, and the time to reach equilibrium may well be more than the hour allowed for primary settling. The concentration of normal globulin molecules involved in nonspecific inhibition will be much larger, and equilibrium will be reached proportionately sooner.

SUMMARY.

The nonspecific inhibitory titres of human sera, against haemagglutination by some influenza virus strains, are strongly influenced by differences amongst the cells obtained from individual fowls.

A relatively small proportion of fowls provides cells which give maximal titres in straight virus titrations, and minimal values for nonspecific inhibitory titres.

A modified form of Salk's technique for titration of influenza antibody is described, depending on the use of fowl cells of this type.

A theoretical treatment of the influence of cell differences on the results of antihaemagglutinin titrations, is included in the discussion.

Acknowledgment. We are much indebted to Miss N. McArthur for help in the theoretical discussion of our results.

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INHIBITORY AND INACTIVATING ACTION OF NORMAL FERRET SERA AGAINST AN INFLUENZA VIRUS STRAIN

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In previous work on the nature of the O-D change in influenza virus, some tests were made to determine whether antigenic differences could be detected between the two phases. Using haemagglutination methods it was found that any differences were very small, the most to be detected being a two-fold difference in favour of the homologous serum.

It was thought possible that immunization with killed O virus might conceivably have an advantage in conferring active immunity beyond that indicated by comparisons of O and D virus *in vitro*, and preliminary experiments in this direction were made in ferrets. The results showed only the same degree of partial protection as is obtained with D strains, and the experiments were not pursued beyond the preliminary stage.

In the course of the work, very high nonspecific inhibitory titres were encountered when normal (pre-inoculation) ferret sera were tested by haemagglutination methods against BEL, O and D virus. The main part of the present paper will deal with the nonspecific effects of normal ferret sera on the two phases of BEL, a strain isolated in 1942, and used as a standard strain for most of our work on O-D changes. In addition to inhibiting haemagglutination it could be shown that all the normal ferret sera tested had well-marked powers to inhibit infection of chick embryos and mice by this strain. The fraction of normal ferret serum which is responsible for this effect has the properties of a gamma globulin.

MATERIALS AND METHODS.

Practically all the work was concerned with the influenza A strain BEL isolated from a pool of throat washings in 1942 and maintained in O phase by high dilution amniotic passage, the more recent passages being made with embryo lung emulsions absorbed with normal fowl erythrocytes. The D phase was maintained by allantoic passage (Burnet and Bull, 1943; Burnet and Stone, 1945a). The other strain used in some experiments was the standard "Melbourne Mouse" (MM) strain, maintained by allantoic passage.

Ferret, mouse and chick embryo emulsions were made by standard methods. Nasal washings were obtained from infected ferrets by the method previously described (Burnet and Bull, 1943). They were titrated by amniotic inoculation with penicillin and sodium sulphadiazine after particles had been removed by paper filtration (Burnet and Stone, 1945b).

Haemagglutinin titrations were made by the method used in this laboratory, the pattern of the deposited cells being used as the index of activity.

EXPERIMENTAL.

FAILURE OF O VACCINE TO IMMUNIZE FERRETS AGAINST HOMOLOGOUS VIRUS.

Two ferrets, 193 and 194, were immunized with embryo lung virus of BEL 0 type with a titre of 500, inactivated with 0·1 p.c. formalin. Two others, 195 and 196, were treated with standard concentrated vaccine made from "Melbourne" with a titre of about 1,500 (10–20,000 by Salk test). Both sets of animals received two weekly injections of 1 ml. vaccine subcutaneously. Two weeks after the last immunizing injection a test bleed was made and then a chal-

[1] This work was carried out under a grant from the National Health and Medical Research Council.

lenge inoculum of BEL O diluted 1:250 was given, two normal ferrets, 197, 198 being included as controls. At least one of each set was tested for the presence and type of virus in the nose at the time of first temperature reaction according to the technique previously described. The washings from the control ferrets 197 and 198 were pooled and titrated in detail in order to show that an O type infection was actually initiated (see Table 2). The antihaemagglutinin titrations against BEL O virus show only insignificant changes as a result of immunization and challenge. Two further ferrets, 199 and 200, were inoculated with BEL O virus diluted 1:1,000, only 0·05 ml. being given intranasally to ensure an O type infection. This was verified by isolation and typing of the virus present in the nose on the third day (initial temperature reaction), as well as on fifth, seventh and ninth days. Table 2 gives the findings, together with those of the pooled washings of ferrets 197 and 198 referred to above. This will indicate that all four ferrets had active O type infections, and in view of results to be given later allows a reasonable certainty that the ferrets had not been spontaneously infected with influenza A before we received them.

TABLE 1.

Reactions of ferrets immunized with O and D influenza virus.

Ferret.	Immunization.	Challenge: Temperature	Challenge: Virus in nose.	Antihaemagglutinin titres against BEL D: I	II	III
193	BEL O c.c.	103–1		800	600	600
194		103·0	10^{-2} 00– –	320	600	600
195	Melb. D Sc.	103·3	10^{-3} 00– –	550	680	700
196		103·6–104·3		960	720	800
197	—	103·5–103·6	10^{-3} 000	480	400	
198	—	104·2		480	600	

Immunization by formalin treated vaccine of type shown.

The temperature given °F. is that of the primary spike; if a secondary peak occurred the height of this is also shown. Under "virus in nose" is shown the result of amniotic inoculation of the highest dilution of nasal washing from which virus was isolated.

0 ♂ or ♂ indicates a positive isolation in the phase shown, – that the embryo inoculated showed no evidence of infection. Antihaemagglutinin titres are given for I preliminary serum, II serum taken 2–3 weeks after immunization, or in the case of 197 and 198, after infection, III serum taken 14 days after the challenge infection.

TABLE 2.

Virus in nasal washings of ferrets infected with Bel O virus.

		Third day.	Fifth day.	Seventh day.	Ninth day.
F197-198	10^{-0}	♂♂♂0			
	10^{-2}	0000			
	10^{-3}	000			
	10^{-4}	0 – –			
F199	10^{-0}	♂♂00	♂♂00	– – –	– – –
	10^{-2}	0 – –			
	10^{-3}	00 – –			
	10^{-4}	000–			
F200	10^{-0}	♂♂♂♂	♂♂0	– – –	– – –
	10^{-2}	000–			
	10^{-3}	00 –			
	10^{-4}	– – –			

Symbols as Table 1.

Finally, two ferrets, F201 and 202 were infected with BEL D and gave a typical monophasic temperature response with maxima of 103·8° F. and 104·3° F. respectively.

Normal serum was thus available from ten ferrets which showed no immunity to either BEL O or D. The reactions of these normal sera are described in the next section.

TABLE 3.

Nonspecific effects of normal ferret sera on Bel O and D.

Anti-haemagglutinin titres of ferret sera.

Ferret.	Normal serum. BEL O	Normal serum. BEL D	Post-infection serum. BEL O	Post-infection serum. BEL D
197	650		1000	360
198	900		1200	
199	240	240	480	240
200	480	480	3200	1000
201	1200	640	1200	640
202	1200	300	1200	600

Tests with BEL O made with guinea-pig red cells, fowl cells were used with BEL D.

When tested in the usual fashion for antihaemagglutinin activity against both phases of BEL, preliminary bleedings showed very high values. which were only slightly, if at all, lower than the post-infection serum samples. Figures for six of the ferrets are given in Table 3. Similar high values for normal ferret sera were obtained against two other O strains of influenza virus A.

Tests were made in which most of these sera were titrated against the two phases of BEL in chick embryos. The D phase experiments were done by the allantoic inoculation method, while O neutralization tests were done using amniotic methods. Owing to the great amount of labour required to inoculate and harvest embryos inoculated by the amniotic route these experiments were limited to those necessary to establish the existence of well marked inactivation by normal serum.

TABLE 4.

Inactivation of O Phase virus by normal ferret sera.

Ferret serum	Results of inoculation of virus diluted 10^{-5} with serum diluted — 1:3	1:10	1:30
199–1	+ — — —	— — — —	— — —
200–1		+ —	— —
201–1	+ — — —		— —
202–1	— — — —		+ + +
Controls:	+ + + —,	+ + + —	

All tests were made by amniotic inoculation + positive haemagglutination by amniotic fluid from an inoculated embryo, — negative result. Two experiments are combined: embryos dying before four days are not recorded.

TABLE 5.

Inactivation of D Phase virus by normal ferret sera (allantoic method).

Serum.	Log activity of serum. Against BEL D.	Against Melb. D.
F199–1	2·9 2·5	—0·5
F200–1	2·7 3·0	—0·3
F197–1	2·0	≤ 1
F198–1	2·5	≤ 1

Figures represent the log of 50 p.c. infective doses inactivated by undiluted serum. See text.

From Table 4 it will be seen that normal sera 199, 200 and 201 show inactivation of a small dose of pure O virus at least to a serum dilution of 1:30. Each serum shows one failure to inactivate at a lower dilution. Normal serum 202 inactivates at 1:3, but not at 1:30.

In Table 5 the activity of four normal ferret sera against the D phase strains Melbourne and BEL is shown. The notation used indicates the log number of infective doses inactivated by undiluted serum when mixed with an equal volume of virus. If a direct titration of tenfold virus dilutions gave a 50 p.c. endpoint $10^{7 \cdot 7}$, while a similar series mixed with undiluted serum

gave an endpoint of $10^{5 \cdot 0}$, then after adjusting the latter figure to $10^{5 \cdot 3}$ to allow for dilution by serum, the activity of the serum is expressed as 2·4.

Owing to the special character of neutralization experiments in the allantoic cavity (Burnet, 1943; Burnet, Stone and Kerr, 1945) the titres expressed in this fashion give a distorted idea of the antibody content of the sera. If as in the previous paper we adopt a value of 1 for a serum which undiluted just neutralizes 10^4 infective doses of virus, then the corresponding values for the neutralization of 10^5, 10^2 and 10 doses will be approximately 0·53, 0·28, 0·15 respectively.

None of the sera shows any detectable activity against Melbourne—all four show a significant approximately equal inactivating effect on BEL. Three of the sera showed a considerable rise against BEL D after immunization with BEL O, the values obtained being 7·5, 7·5, 6·3, while ferret 197 showed no rise with a value of 2·6

The strain BEL D has only slight pathogenicity for mice but produces visible lung lesions to a dilution of 1:100. Tests with normal ferret sera 199, 200 and 202 all showed definite inactivation beyond a serum dilution of 1:10 (Table 6).

TABLE 6.

Inactivation of BEL D for mice by normal ferret sera.

	1:10 virus + serum diluted.						
Serum.	Undiluted	1:10	1:100		Virus control + saline.		
199	0000	0000	011—1—	(0·5)	1:10	2211—	(1·4)
200	0000	0000	2111—	(1·1)	1:100	1111—	(0·9)
201	xx00	x000	11—1—0	(0·5)	1:1000	111—0	(0·6)
202	000x	0000	11—00	(0·4)			

Mouse lung lesions were all incomplete and are graded according to standard convention, 2 representing consolidation of $\frac{1}{6}$–$\frac{1}{2}$ the lung surface. 1 and 1— smaller degrees of consolidation. 0 no lesions. x nonspecific lesions or death.

The average intensity of the lesions is shown in brackets.

THE NATURE OF THE INHIBITORY SUBSTANCE IN NORMAL FERRET SERUM.

In view of this evidence of unusual activity of a normal component in ferret serum, which not only inhibited haemagglutination to high titre but had in addition power to neutralize the strain BEL *in vivo*, limited studies were made of its physicochemical character. This work was carried out by one of us (J.F.McC.) concurrently with a more extensive investigation of the nonspecific inhibitor of haemagglutination present in normal rabbit serum (McCrea, 1946). The fractionation methods used are fully described in that paper.

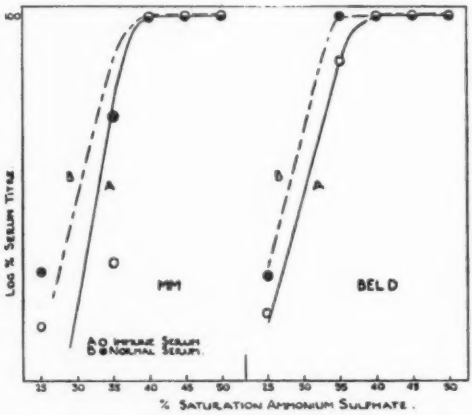

Fig. 1. Recovery of specific and nonspecific inhibitory activity in ferret serum fractions (ammonium sulphate).

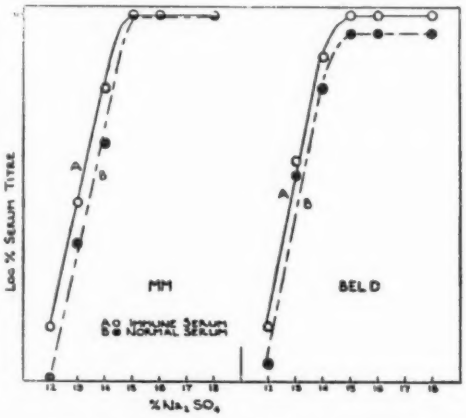

Fig. 2. Recovery of specific and nonspecific inhibitory activity in ferret serum fractions (sodium sulphate).

Fractionation of Normal and Immune Ferret Sera with Ammonium and Sodium Sulphates.

When either normal or immune diluted serum was treated at 50 p.c. saturation with ammonium sulphate, all the inhibitory activity was concentrated in the precipitate. Reprecipitation of the dissolved material at 33 p.c. saturation, which was effective in precipitating most of the activity from rabbit serum, however, gave very little precipitate and little inhibitory activity was carried down.

Serial fractionation from undiluted serum was carried out with both normal and immune serum the successive precipitates being redissolved in saline and tested against both MM and BEL D virus.

The results shown in Fig. 1 indicate that all of the nonspecific inhibitor of BEL is precipitated at 33 p.c. saturation, whilst a much smaller proportion of the specific or nonspecific activity against MM is precipitated at this concentration. All activity is precipitated at 40 p.c. saturation.

Ferret serum also behaves rather atypically on sodium sulphate fractionation, but nevertheless reasonably satisfactory curves can be obtained with careful control of pH and other conditions.

The results from both series of experiments indicate that both normal and immune inhibitors are probably present in the gamma globulin fraction, as is the case with rabbit sera. It is of interest that the normal inhibitor of BEL D haemagglutination is precipitated at a slightly lower salt concentration than the inhibitor for strain MM.

Heat Inactivation of Inhibitors in Normal Ferret Serum.

In normal rabbit serum the inhibitory effect is rapidly destroyed at 62° C., while immune antibody is very little affected. On applying the same test to normal ferret serum the results were found to depend entirely on whether the nonspecific inhibitory effect was measured with BEL or MM (Table 7).

Table 7 shows that the power to inhibit MM haemagglutination is completely lost within 20 minutes but that the inhibitory power against BEL is much more resistant, behaving in fact very like an immune antibody.

TABLE 7.

Heat inactivation of normal ferret serum at 62° C.

	MM.		BEL D.	
Time (Minutes).	Antihaem-agglutination titre.	P.c. control.	Antihaem-agglutination titre.	P.c. control.
0	560	—	560	—
10	35	6	240	43
20	<10	—	160	29
30	<10	—	80	14
40	<10	—	60	11

DISCUSSION.

These observations appear to establish, *first*, that the high inhibitory power of normal ferret serum against haemagglutination by a strain BEL of influenza A is not a result of previous unrecognized infection. This is shown, both by the ability of the strain to infect the ferrets typically in small dose, and by the fact that the sera have no effect *in vivo* whatever, against the strain "Melbourne" which by other criteria, is serologically closely related to BEL. *Second*, the power to inhibit haemagglutination is associated with a low but quite distinct power to inactivate BEL in tests in the allantoic cavity and by intranasal inoculation in mice. It is reasonable to assume that all three activities are related to the same component of the sera.

Fractionation by salt precipitation methods indicates that the component in question forms part of the gamma globulin fraction of the serum proteins. It appears to be precipitated slightly more readily than either immune globulin or the fraction of normal serum responsible for inhibiting "Melb." haemagglutination. Its resistance to heat is markedly superior to that of the latter fraction resembling in this respect that of a specific antibody.

The experiments indicate a possible source of error in the interpretation of serological reactions with influenza viruses and provide a new example of pseudo-immunological activity of a normal serum component. It is probable that the phenomenon is essentially similar to the well-known instances in which normal sera agglutinate or haemolyse cells from a heterologous species. Tyler and Metz (1945) have recently described a striking example in showing that crayfish serum can agglutinate almost the whole range of vertebrate blood cells. Absorption experiments showed that several different components of the crustacean serum were involved.

In these non-immune reactions a proportion of serum globulin molecules appear to possess certain surface configurations, which are capable of reacting with complementary type configurations on the cells and virus particles concerned, the forces involved probably being of essentially the same character as those responsible for antigen-antibody union. In the present state of knowledge it is probably a matter of simple chance whether the denaturing effect of heating in the region of 62° C. disturbs the globulin configuration sufficiently to destroy the non-specific reactivity, as is the rule, or leaves it still effective as is the case with the BEL inhibitor in the present work.

SUMMARY.

In studies of the possible immunological significance of the O-D phase difference, the strain BEL in the O and D phases were used to prepare ferret anti-sera.

Attempts to immunize ferrets with O phase virus were not significantly more successful than with standard D phase virus. Multiplication of virus in the nasal cavity was demonstrated in immunized ferrets.

Haemagglutination titrations of immune ferret sera were rendered unsatisfactory because of the very high inhibitory activity of the sera before infection or immunization. This inhibitory effect in normal serum was particularly great against BEL O and D virus.

Several "normal" ferret sera were shown to inactivate virus by tests in chick embryos and mice. The "neutralizing" activity against O virus was particularly marked in amniotic inoculation. The inhibitory component of normal ferret sera active against BEL O and D was more heat resistant than the same component tested against MM. In this respect the inhibitory activity against BEL resembles that of specific antibody, rather than the normal inhibitory component against other virus strains.

On serum fractionation the normal and immune inhibitory activities are recovered similarly, suggesting that the normal inhibitory component also is a gamma globulin.

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NONSPECIFIC SERUM INHIBITION OF INFLUENZA HAEMAGGLUTINATION

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The application of the Hirst haemagglutination reaction to the titration of virus antisera has marked a great advance on methods previously available, since with this technique it is possible to obtain results in one hour, which by previous methods could be obtained only after several days and at the expense of considerable experimental material. However, one limitation to the widespread application of the method has been the presence in many normal sera of a substance inhibitory to the influenza haemagglutinin. This substance in normal serum inhibits haemagglutination in an apparently similar manner to specific antibody and the inhibition in normal ferret and rabbit serum, for example, may extend to a dilution of the order of one in several hundred. The inhibitory titre of normal animal (and human) sera varies greatly with the virus strain used for testing, the strain BEL D giving particularly high titre with most sera. Typical values for normal rabbit serum with this strain vary from 100 to 320. Such nonspecific effects present obvious difficulties in interpreting results where the previous clinical history of a serum is doubtful or unknown. Further, the presence of both specific and nonspecific inhibition in the one serum is a serious complication in experimental work with laboratory animals.

This paper describes a simple heat treatment which rapidly destroys the nonspecific inhibition in rabbit serum, leaving the true immune inhibition relatively unchanged. It is thus possible accurately to titrate an immune serum without interference from the nonspecific factor when both are present in the one serum, and also to differentiate between a serum giving immune neutralization and one giving "normal" neutralization only.

The isolation of the inhibitor from normal rabbit sera is described, and its behaviour compared with that of the inhibitor isolated from immune sera by the same methods. Similar studies on ferret serum are described elsewhere (Burnet and McCrea, 1946).

TECHNIQUES.

Bleeding and Inoculation.

Blood was collected with aseptic precautions from the marginal ear vein of adult rabbits, and the serum removed several hours later from the clot. At least three weeks were allowed to elapse between successive bleedings from any one animal, after which the sera were pooled and stored without preservative at 4° C.

Rabbits were immunized by an intravenous injection of 1 ml. undiluted BEL D allantoic fluid, a second similar injection being given 10 days later. The strain BEL D was used throughout because of its unusually high sensitivity to nonspecific serum inhibition. It is an influenza A strain isolated in 1942.

Stock viruses were prepared by the standard suballantoic method now used in this laboratory (Burnet and Bull, 1943) and passaged twice from seed virus maintained at —15° C.

[1]This work was carried out under a grant from the National Health and Medical Research Council.

Haemagglutinin Titrations.

Virus and serum neutralization titrations were carried out following the standard method described by Burnet (1942). Serum and serum fractions were diluted in calcium saline to give a series of doubling dilutions of 0·25 ml., 0·25 ml. of virus diluted to 5 agglutinating doses was added, and finally 0·25 ml. of a 2 p.c. fowl red cell suspension. The tubes were read after standing at room temperature for 75–90 minutes. In a series of comparative estimations, as for example, in the titration of remaining inhibitory activity in sera heated for various times or the recovery of activity in various fractions from sera before and after immunization, particular care was taken to standardize the conditions by carrying out all tests in the series at the same time, and using the same dilution of virus and the same red cell suspension. When heated sera or sera fractions were being titrated the original sera were always titrated at the same time.

Protein Estimations.

Protein concentration of serum fractions was estimated by determining tyrosine colorimetrically with Folin-Ciocalteu phenol reagent (Folin and Ciocalteu, 1927).

Fractionation Methods.

As electrophoresis equipment is not at present available, all fractionation procedures were necessarily based on the older salt-fractionation methods. Recent revision and extensions of these methods have shown reasonably close correlation between serum fractions prepared by electrophoresis and by salt-precipitation under standardized conditions (Cohn, McMeekin, *et al.*, 1940).

Ammonium sulphate. Saturated ammonium sulphate solution was prepared from Merck's analytical reagent. The pH of such a solution varies from 4·5–6·5, but was not readjusted as fractionation was carried out at a final serum-salt mixture pH of 6·0–6·5. All pH measurements were carried out with the glass electrode.

Following recent practice the serum was not diluted, or at most diluted with an equal volume of distilled water before adding the ammonium sulphate. The procedure was based on that of Cohn, McMeekin *et al.* (1940), with the exception that the ammonium sulphate was added dropwise at 0° C. with stirring instead of being dialyzed in through a cellophane membrane. As a routine the serum-salt mixtures were left overnight at 0° C. before separating the precipitate, although satisfactory recovery could usually be obtained after standing for 1–2 hours at that temperature.

In preliminary experiments described below, the precipitates were removed by filtration on Whatman paper number 542 with gentle suction on Hirsch funnels. One filtration, returning the first few ml. was generally satisfactory for rabbit serum, but several passages were necessary for ferret serum. Although some error is introduced through absorption of protein by the paper (Robinson *et al.*, 1937), this was not considered sufficient to affect the accuracy limitations of the experiments. The precipitates were washed through the paper in repeated small quantities of 2 p.c. saline and made up to volume in 2 p.c. saline. In later experiments it was found more convenient to centrifuge down the precipitates in a high-speed "Ecco" centrifuge; after centrifuging at 12,000 r.p.m. for 20 minutes a firm cohesive deposit was obtained from which the supernatant fluid could be decanted cleanly. The cups were then inverted to drain for several minutes, the sides wiped dry, and the precipitate made up to the original serum volume in 2 p.c. saline. Except when otherwise stated, the precipitates were washed once with ammonium sulphate at the precipitation concentration.

Sodium sulphate. The method here essentially followed that described by Howe (1921) and Robinson *et al.* (1937). Solutions of the required concentration were prepared and stored at 35° C., the fractions being precipitated at the same temperature and at a final serum dilution of 1:30. The final pH of the serum-salt mixtures was controlled at 6·5–7 by adding sufficient sodium bisulphate to the sodium sulphate solutions during their preparation to bring the pH to approximately 5·5; the amount necessary (of the order of a fraction of a ml. of 10 p.c. $NaHSO_4$ solution per 100 ml. sodium sulphate solution) was determined separately by preliminary trial for each serum and salt concentration, as it was found that the buffering capacity of sera varies slightly. Precipitates were left for at least four hours before being separated by filtration or centrifugation as described previously, washed once with the corresponding sodium sulphate solution and made up to the original serum volume in 2 p.c. saline.

All fractions were stored at 4° C. except when in immediate use.

EXPERIMENTAL.

HEAT INACTIVATION OF NORMAL RABBIT SERUM INHIBITOR.

As a preliminary, 1 ml. of uninactivated serum from each of four normal rabbits was heated for 30 minutes in a water bath at 55°, 65°, 75° and 85° C. Little change in opacity or viscosity was noticeable up to 65° C., but heavy coagulation occurred at 75° and 85° C.; exuded fluid from the latter was obtained for titration after macerating and standing overnight.

Titrations of these sera with BEL D showed an average inhibition reduction of 50 p.c. at 55° C. compared with untreated sera, but complete destruction of inhibitory activity was obtained from 65° C. and upwards, i.e. before coagulation of the serum proteins takes place.

More accurate determinations were then made by heating approximately 0·2 ml. of serum in sealed glass tubes immersed in a water bath equipped with stirrer and controlled to ± 0·1° C. These showed that the critical temperature for destruction lay between 60°–64° C. (Table 1) with only slight variation between individual sera.

TABLE 1.

Destruction of normal inhibitory activity after 30 minutes at various temperatures.

Serum 118, BEL D 99058, Fowl 83.

Temperature °C.	Dilution of serum. 10	20	40	80	160	320	Titre.	P.c. control
Unheated	0	0	0	0	0·5	1·5*	200	—
56	0	0	0	0·5	1·2	1·5	140	70
58	0	0	1·2	1·8	2·0	2·0	35	18
60	0	1·2	2·0	2·0	2·0	2·0	18	9
62	1·5	2·0	2·0	2·0	2·0	2·0	<10	—
64	2·0	2·0	2·0	2·0	2·0	2·0	<10	—

* Tubes were read thus: 2·0 = complete agglutination.
1·0 = endpoint at partial agglutination.
0 = no agglutination.

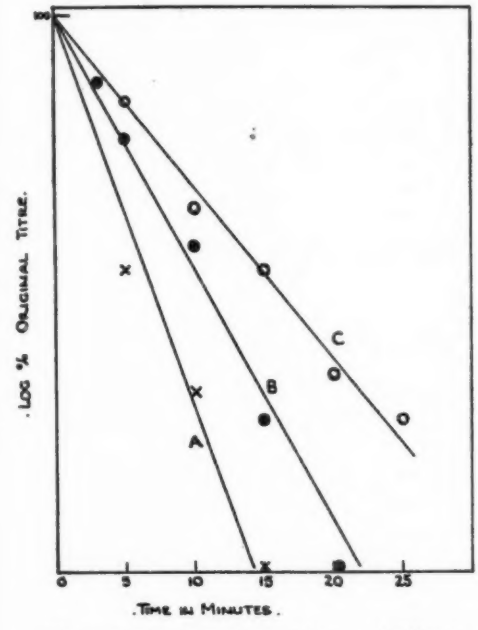

Fig. 1. Destruction of normal serum inhibitory activity at 62° C. A=serum 116, B=serum 117, C=serum 118. All sera first bleeds from young adult rabbits. Ordinates express serum neutralization titre as log of the percentage of original unheated titre.

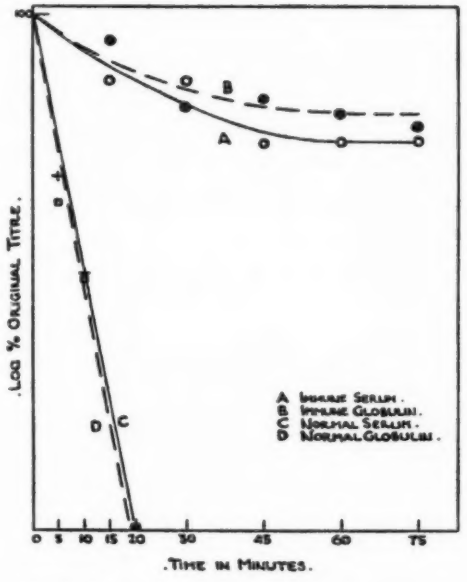

Fig. 2. Destruction of specific and nonspecific inhibitory activity at 62° C.

Destruction at 62° C.

Samples from all available normal sera were then heated for 30 minutes at 62° C. In the great majority of sera this resulted in complete loss of nonspecific inhibition, those sera not being completely inactivated showing at most approximately 5 p.c. of the original inhibition. To investigate the rate of destruction at 62° C., and also to show more clearly the variation between individual sera, approximately 0·5 ml. samples were sealed in short lengths of glass tubing and immersed at 62° C. ∓ 0·1 for various times; two minutes were allowed for the sera to reach bath temperature and they were cooled immediately on removal from the bath. Fig. 1 shows typical curves so obtained.

It will be seen that the inhibitory activity falls off logarithmically with time, similarly to the curves obtained in the original heat disinfection studies of Chick (1910) on typhoid bacilli, and indicating also that the limiting inactivation reaction or reactions are probably of the first order.

Most sera tested behave as shown in curve B, the inactivation proceeding steadily until inhibition is no longer detectable at 20 minutes. Occasionally sera are found in which the inhibitory activity is destroyed more rapidly (curve A); or in which the activity is initially destroyed logarithmically, but thereafter more slowly with approximately 5 p.c. of the original titre persisting for 30–40 minutes (curve C). The variation in the rate of destruction is characteristic of each serum, and can be reproduced at different temperatures, but it does not appear to be connected with the age or breed of rabbit.

Comparative Destruction Rates at Different Temperatures.

A great increase in the rate of destruction takes place with the relatively small temperature increase of 6° C. between 56° and 62° C. (Table 1). Further trials at higher temperatures up to the coagulation point showed a continual increase in destruction rate with, for example, complete destruction in five minutes or less at 65° C. However, for practical purposes the relatively slower inactivation at 62° C. for 20 minutes seems safer and is the standard method used in this laboratory for inactivating rabbit sera.

In an attempt to estimate the approximate temperature coefficient of the destruction, samples were removed from the bath at intervals after heating at 60° and 64° C. and titrated as described previously. Although slightly higher at the beginning of the destruction, the velocity constants showed reasonable constancy considering the accuracy limitations of the method of estimation, and the curves of rate of destruction closely approach straight lines when plotted logarithmically against time. The mean velocity constants are 0·050 ± ·005 and 0·111 ± ·005 at 60° and 64° C. respectively. The temperature coefficient is 2·2 for a 4° C. rise, or roughly 6 for a 10° C. rise, assuming that the increased rate of destruction is uniform over the temperature scale.

Comparative Heat Stability of Normal and Immune Inhibitors.

Sera from rabbits before and after immunization were heated at 62° C. and samples for titration removed at intervals as described previously. Results for rabbit 48 are shown in Table 2.

TABLE 2.

Heat stability of serum at 62° C. before and after immunization.

Time (minutes).	Before immunization. Titre.	Before immunization. P.c. control.	After immunization. Titre.	After immunization. P.c. control.
0	160	—	320	—
5	40	25	280	90
10	<10	—	240	75
15	<10	—	160	50
20	<10	—	160	50

The destruction rate in the immune serum is obviously of a different order from that in normal serum; at the time when the nonspecific inhibitory titre is completely destroyed, 75 p.c. of the immune titre remains and thereafter falls more slowly until it reaches an almost constant level at 30–40 p.c. of the original titre (Fig. 2). During the second hour the immune titre falls only a further 5 p.c. This resistance to heat inactivation is characteristic of all immune sera tested, and no decrease in resistance has been observed after storage at 4° C. for several months.

FRACTIONATION OF RABBIT SERUM.

Ammonium Sulphate.

Table 3 shows recovery of immune and nonspecific inhibition in rabbit serum fractions. Total globulin was first precipitated (Fraction I) at 50 p.c. saturation, redissolved in saline and

brought to 33 p.c. saturation; this precipitate (II) was also filtered off and redissolved, the filtrate brought to 50 p.c. saturation and the precipitate (III) removed and redissolved as before. Albumin was precipitated with dilute acetic acid from the filtrate from III and redissolved in saline. The fractions were titrated by diluting out in exactly the same way as for the sera, control titrations in the presence of ammonium sulphate (as in the filtrates) showing no interference with the test. The virus used throughout was BEL D 99058 diluted to 5 agglutinating doses.

TABLE 3.

Recovery of specific and nonspecific inhibitory activity from rabbit sera.

Fraction.	P.c. saturation ammonium sulphate.	Serum.	Titre.	P.c. original serum titre.
Whole serum	—	Normal	240	—
		Immune	480	—
I Total globulin	50	Normal	190	80
		Immune	400	85
II Gamma globulin	33	Normal	220	90
		Immnue	420	90
III Alpha and beta globulin	33–50	Normal	2	<1
		Immune	16	3
IV Albumin		Normal	<2	<1
		Immune	<2	<1

From the Table it is at once apparent that both specific and nonspecific inhibitors are recoverable in the globulin fraction. Redissolved albumin and the filtrates from Fraction I–IV gave no demonstrable inhibition of agglutination. When the total globulin is refractionated, the inhibitory activity is almost quantitatively recovered in the least soluble globulin fractions precipitated up to 33 p.c. saturation, which, assuming that the immune serum containing typical antibodies may be taken as a standard, can be designated gamma-globulins. The remaining globulin fractions (alpha and beta globulins) contain only negligible amounts of the inhibitors, probably due to traces of contaminant gamma globulin.

When the various fractions are reprecipitated once more at the same respective concentrations of ammonium sulphate, 60–70 p.c. of the original normal and immune inhibition is recovered in the gamma globulin fractions, whilst the recovery in the alpha and beta globulins becomes almost undetectable. Further reprecipitation of the gamma globulins yields progressively less precipitate and lower recovery of inhibition. Progressively higher concentrations of salt are necessary completely to reprecipitate from relatively pure solutions of one component (Howe, 1921).

Direct precipitation of the gamma globulins by 33–40 p.c. saturation, without first precipitating total globulin, yields essentially similar recovery of inhibitory activity. The characteristic difference in heat stability of the normal and immune sera is retained by the globulins after precipitation and redissolving, as is shown in Table 4.

TABLE 4.

Heat stability of inhibitors from serum fractions at 62° C.—15 minutes.

Fraction.	Serum	Treatment.	Titre.	P.c. original titre
Total globulin	Normal	Control	128	
Total globulin	Normal	Heated	4	3
Total globulin	Immune	Control	256	
Total globulin	Immune	Heated	128	50
Gamma globulin	Normal	Control	96	
Gamma globulin	Normal	Heated	6	6
Gamma globulin	Immune	Control	320	
Gamma globulin	Immune	Heated	160	50

The rate of destruction of inhibitory activity in the various once-washed globulins is also apparently similar to that in the original sera. This is shown in greater detail in Fig. 2.

Serological Relations of the Fractions.

Results of titrating sera and their respective fractions for inhibition of strains BEL D, LEE B and WS are given in Table 5. These three strains were chosen because they exhibit respectively extreme, intermediate and minimal sensitivity to nonspecific inhibition.

TABLE 5.

Inhibition titre of sera and fractions with BEL D, LEE B and WS.

		Serum.	Total globulin.	Gamma globulin.
BEL D	Normal	240	190	220
BEL D	Immune*	480	400	360
LEE B	Normal	280	90	60
LEE B	Immune*	280	90	60
WS	Normal	0	0	0
WS	Immune*	30	0	0

* Immune serum to BEL D. 99058.

It will be seen that the serological relationships of the viruses are retained when titrated with the redissolved total and gamma globulins; with LEE B the recovery of inhibition in the globulins is lower than with BEL D, but the ratio of inhibition in normal and immune sera and fractions remains constant. The inhibition titres against LEE B are, of course, non-specific with all sera and fractions, but the titre of 30 with immune serum against WS is probably a cross reaction with true BEL D antibody, both viruses being strains of influenza A. No inhibitor was demonstrable with the redissolved alpha and beta globulins.

Sodium Sulphate.

Keckwick (1940) and Keckwick and Record (1941) have recently described serum fractionation methods with sodium sulphate which yield reasonably pure preparations of globulins. Total globulin is precipitated by adding 18 p.c. Na_2SO_4 directly to serum, and gamma globulin by adding 12 p.c. Na_2SO_4 to the total globulin redissolved in 40 p.c. of the original serum volume in phosphate buffer at pH 8. Results obtained following this procedure are given in Table 6.

TABLE 6.

Keckwick fractionation of normal and immune rabbit serum.

Fraction.	Normal titre.	P.c. recovery.	Immune titre.	P.c. recovery.
Whole serum	280	—	1,120	—
Total globulin	240	85	960	85
Gamma globulin	200	70	960	85

Here again both normal and immune inhibitory actions are recovered satisfactorily in the gamma globulin fractions. The titre of all supernatant fluids after the removal of the precipitates was negligible, indicating that the slight loss of titre in the fractions is probably due to protein denaturation during the process.

Serial Fractionation.

Comparative salting-out curves of inhibition recovery from normal and immune sera were constructed using the slight modification of Howe's fractionation method described previously. Essentially similar results were obtained with either filtration or centrifugation. Each point on the figures represents the logarithm of the inhibition of the globulin fraction as per cent. of the original serum inhibition.

It is immediately apparent from Fig. 3 that the normal serum inhibitor and the immune inhibitor are salted-out identically. Recovery of inhibitory activity increases at the same rate in both sera, and is complete at the same sodium sulphate concentration. This is taken as being further evidence that the nonspecific inhibition is due to normal serum globulin. Full recovery of inhibitory activity occurs at 14 p.c. sodium sulphate, although the protein precipitated obviously continues to increase with sodium sulphate concentration; globulin estimations confirmed a steady increase in concentration, continuing past the point of complete inhibition recovery.

MECHANISM OF HEAT INACTIVATION.

It has not been possible to carry out a thorough investigation on the mechanism of the heat inactivation of the nonspecific inhibitory activity of serum, but the following experiments were made. To discover any solubility change accompanying possible denaturation of the protein, 5 ml. of normal serum were heated at 62° C. until the nonspecific inhibitory effect was just destroyed. The pH of the serum was then adjusted to 4·5 with 0·1 N HCl. A fairly intense opalescence results, but untreated serum adjusted to the same pH shows no change in opacity. When immune serum is heated for the same time and at the same temperature and adjusted to pH 4·5, some change in opacity is apparent when compared with unheated serum at the same pH, but the change is obviously less than in normal serum.

A further experiment suggests that at least part of the heat inactivation in normal sera is due to complex-formation with serum albumin. The inhibitory titre of unwashed or once-washed normal globulin is destroyed by heating at 62° C. at the same rate as in the original serum (Fig. 2), but after several further washings the globulin becomes more heat-resistant. Fig. 4 shows relative heat-inactivation rates of a normal serum, the serum globulin precipitated and redissolved in saline without washing, and part of the same globulin thoroughly washed four times in the corresponding sodium sulphate solution.

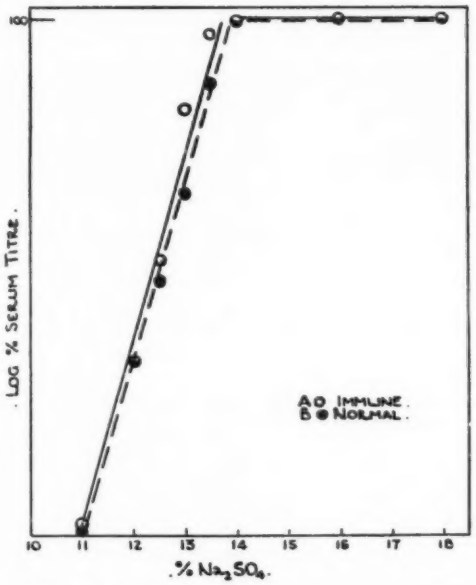

Fig. 3. Recovery of specific and nonspecific inhibitory activity in serum globulin fractions.

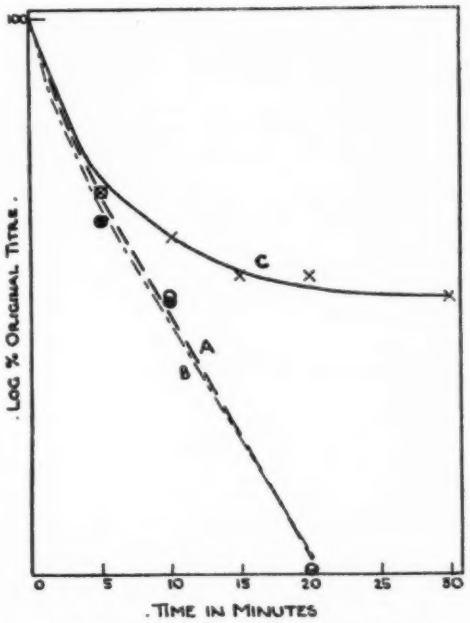

Fig. 4. Destruction at 62° C. of the nonspecific inhibitory activity of normal serum, the unwashed serum globulin, and the globulin washed four times. A=serum, B=globulin, C=washed globulin.

Assuming that the function of repeated washing is to remove the last traces of serum albumin, it is clear that some interaction between the serum globulin and albumin must be responsible for the final stages of heat inactivation in normal rabbit serum. Initially the rate of destruction in serum and in washed globulin is similar, but thereafter destruction in the latter becomes slower, tending to remain constant from shortly before the time that inhibitory activity of whole serum and unwashed globulin is destroyed. The initial fall is probably associated with preliminary denaturation before complex formation.

DISCUSSION.

It is of particular interest that a normal serum can behave in some respects in a way immunologically similar to virus antisera; and it becomes even more so when it is shown that this behaviour is due to the normal serum gamma globulin.

It is now accepted that with insignificant exceptions specific antibody in immune serum forms part of the gamma globulin. The fractionation experiments described in this paper establish clearly that normal rabbit globulin is also responsible for non-specific inhibition of certain influenza virus haemagglutinins.

Strictly speaking, it is only justifiable to characterize a serum fraction as

gamma globulin if it has been subjected to electrophoretic examination. Nevertheless recent work by Cohn, McMeekin *et al.* (1940), and Keckwick (1940) has indicated that close correlation exists between fractions obtained electrophoretically and those obtained by salt fractionation under controlled conditions. The fact that in our own experiments the active fraction of immune serum was precipitated at the same salt-concentration as precipitated the nonspecific inhibitor, supports the identification of the latter as gamma globulin; and further the salt concentrations found to give complete recovery of both inhibitors are similar to those found by other workers to give complete precipitation of electrophoretically-identified gamma-globulin. In addition it has been shown that the untreated serum and the isolated, unwashed or once-washed globulin fractions behave similarly on heating, and have a similar range of action on heterologous virus strains.

Apparently the surface configuration of normal rabbit globulin is in certain respects sufficiently similar to the configuration of BEL D immune globulin to give a pseudo-specific interaction with the virus antigen. In the absence of detailed knowledge of the physico-chemical differences between normal and immune globulins it is not possible to speculate on the mechanism of this effect.

The second point of interest arising from the work is that in rabbit serum the change from normal globulin to immune globulin is accompanied by an increase in resistance to heat inactivation. It is possible that in immune globulin the surface structure is more stable, and tends to resist changes leading to the loss of characteristic immunological properties. A rather similar effect described for *B. typhosus* antisera is the increase in the resistance of the antibody to enzymatic destruction, which develops with increasing immunity (Rosenheim, 1937).

With the isolation of a normal globulin having certain pseudo-immunological properties, but possessing also certain well-defined differences in heat-stability, it should be possible to demonstrate physico-chemical differences between normal and antibody proteins. It has not been possible to undertake a thorough investigation of this possibility here, but such work as has been done suggests that protein denaturation (judged by solubility changes at the iso-electric point) and complex formation with albumin are involved in the destruction of nonspecific inhibition in normal rabbit serum.

Kleczkowski (1941) has described similar effects on heating plant virus antisera from rabbits at 70°–80° C., when the antibodies combine with albumin to form a complex no longer capable of flocculating the antigen, although still capable of combining with it. He also describes the formation of euglobulin-albumin complex in normal rabbit serum heated to 70°–80° C., the complex being formed from the two components during denaturation and precipitating characteristically with ammonium sulphate. Further, reactive-SH groups appear in heated rabbit euglobulin and euglobulin-albumin mixtures.

This corresponds with the behaviour of rabbit serum heated at 60°–65° C. until the nonspecific inhibition of influenza haemagglutination is destroyed, with the exception that the results of attempts to demonstrate reactive-SH groups by the nitroprusside test were inconsistent in both serum and globulin. One serum in which the nonspecific inhibition was destroyed unusually rapidly (in less than five minutes at 62° C.) gave a faintly positive nitroprusside test, but repeated trials with other sera and globulins were negative. This is probably due to the lower temperature of inactivation used, which may be insufficient to cause denaturation to the stage of the appearance of reactive-SH groups; however, some denaturation occurs, as judged by solubility changes at the isoelectric point.

Thus it appears that thermal destruction of nonspecific inhibition of influenza haemagglutinins and the destruction of flocculating power in rabbit antisera against plant viruses is brought about by a similar mechanism, namely preliminary denaturation of the globulin, followed by complex formation with serum albumin. The antibody globulin in influenza antisera shows no evidence of these reactions at temperatures which rapidly affect the globulin responsible for the haemagglutinin inhibition of normal sera.

Fractionation of ferret serum by the same methods yielded essentially similar

results regarding the identity of normal globulin with the non-specific inhibitor of BEL D, but inactivation experiments showed that the normal globulin behaved surprisingly like an immune antibody. In view of immunological investigations by Professor F. M. Burnet on the action of normal and immune ferret sera on BEL D, these results will be discussed later in a joint paper.

SUMMARY.

The agent in normal rabbit serum responsible for the inhibition of influenza virus haemagglutination is destroyed by heating for 15–20 minutes at 62° C.

The agent in BEL D rabbit anti-serum responsible for true immune inhibition of haemagglutination is relatively thermostable; 30–50 p.c. of the original serum titre remains after heating at 62° C. for 3 hours.

On fractionation with ammonium sulphate and with sodium sulphate the redissolved gamma globulin fractions of both normal and immune sera retain 70–100 p.c. of the original serum inhibitory titre, and show the characteristic heat stability and serological relations of the sera from which they were derived. No significant inhibition is demonstrable in the redissolved alpha and beta globulins.

The thermal destruction of inhibitory activity in normal rabbit serum is accompanied by denaturation of the globulin and complex formation between the denatured globulin and albumin.

Acknowledgment. I am greatly indebted to Professor F. M. Burnet for guidance throughout this work.

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SOME ASPECTS OF THE IMMUNOLOGY OF THE Rh FACTOR

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Levine, Burnham, Vogel and Katzin (1941) first drew attention to the Rh factor in relation to the aetiology of Haemolytic Disease of the Newborn and postulated the theory of maternal iso-immunization during pregnancy to explain the mechanism of its action. They found the anti-Rh agglutinin in the serum of 30 p.c. of 141 mothers of children suffering from Haemolytic Disease of the Newborn. Many workers have since reported its presence in a varying percentage of the mothers of affected infants. The failure to detect the agglutinin in all cases in which iso-immunization is strongly suspected has been confusing. Recent work has, however, revealed the complexity of the immune anti-Rh agglutinin and has provided new techniques for demonstrating evidence of iso-immunization.

Whilst investigating the serum from mothers of diseased children, Wiener (1944) noted that, in many instances, Rh-positive cells showed no agglutination when mixed with the serum, but that the subsequent addition of a known anti-Rh serum did not produce agglutination. He demonstrated that this "blocking" effect was a phenomenon specific to the Rh_0 (Δ) sub-group and that its detection indicated iso-immunization. Race (1944a) independently observed the same phenomenon and termed the new antibody the "incomplete" antibody (as opposed to the complete or agglutinating antibody). The finding of the "blocking" or "incomplete" antibody in the serum of a mother provides proof of iso-immunization. The technique employed by Wiener has been considerably modified since the publication of his original paper. Diamond and Abelson (1945) showed that if a serum containing the "incomplete" antibody was mixed with a 50 p.c. suspension of Group O Rh-positive red cells agglutination was produced. Wiener (1945a) found that clumping of red cells resulted if the cells were suspended in serum, and suggested that this clumping was "conglutination" and due to the presence of unidentified "glutinins" acting only in undiluted serum. Diamond and Denton (1945) found that suspensions of Rh-positive red cells in albumin were similarly agglutinated by the "incomplete" antibody. Coombs, Mourant and Race (1945) have devised a test (referred to in this paper as the "C.M.R." test) which discloses the presence of an incomplete or a weak complete agglutinin on the surface of the red cell. Antibodies are contained in the globulin fraction of serum and when an antibody meets with its specific agglutinogen on the red cell, the antibody cannot readily be removed by washing the red cells with saline. The addition of a serum, obtained from a rabbit immunized against human globulin or human serum, will agglutinate red cells which have been sensitized, and thus have antibody globulin adsorbed at some points of their surface.

All the above tests have been employed in the present investigation, with a number of different sera from persons thought to have been immunized against the Rh factor. The results recorded in this paper demonstrate the relative value of the various tests and show the necessity for complete examination of every sample of serum. An attempt will be made to explain the mechanism of the reactions.

The terminology as used in the scheme worked out by Fisher in 1944 (Race 1944b) is given in brackets after the more widely accepted terminology.

MATERIALS AND METHODS.

Blood for examination was obtained in each instance from the mother of an infant which displayed evidence of Haemolytic Disease of the Newborn. After clotting, the serum was separated and used for the various tests without inactivation.

Red blood cells were obtained from individuals known to belong to the various Rh subgroups. A few drops of blood, obtained by pricking the finger, were placed in a small quantity of 2·6 p.c. solution of sodium citrate, and the suspension diluted with normal saline to provide a 0·5 p.c. suspension (packed volume) of red cells as judged by the eye.

Determination of Rh subgroups. For part of the work there were available sera of the types anti-Rh_0 (Δ), anti-Rh′ (Γ) and anti-Rh″ (H). With these sera the Rh subgroups of the red cells were determined, using the technique described by one of us (Walsh, 1945). However, for the earlier part of the work the sera available were of the anti-Rh_0′ ($\Delta\Gamma$) and anti-Rh_0″ (ΔH) varieties. Artificial anti-Rh′ (Γ) and anti-Rh″ (H) agglutinins were prepared from these sera by mixing equal parts with a serum containing "incomplete" antibody (Δ') in high titre. The results obtained with these mixtures were not nearly as clear-cut as were those with the natural agglutinins. The interpretation of the results was as shown in Table 1. It can be seen that it is impossible to distinguish the very rare Rh′ Rh″ $\left(\frac{Cde}{cdE}\right)$ from the Rh_1 Rh_2 $\left(\frac{CDe}{cDE}\right)$ subgroup by this method.

TABLE 1.

Reactions of red cells of the Rh subgroups with various anti-Rh sera.

Subgroup.	Anti-Rh_0′ $\Delta\Gamma$	Anti-Rh_0″ ΔH	Artificial Anti-Rh′ Γ	Artificial Anti-Rh″ H
Rh_0 (cDe)	+	+	—	—
Rh_1 (CDe)	+	+	+	—
Rh_2 (cDE)	+	+	—	+
Rh_1 Rh_2 $\left(\frac{CDe}{cDE}\right)$	+	+	+	+
Rh′ (Cde)	+	—	+	—
Rh″ (cdE)	—	+	—	+
Rh′ Rh″ $\left(\frac{Cde}{cdE}\right)$	+	+	+	+
rh rh (cde)	—	—	—	—

+ = agglutination. — = no agglutination.

Examination of Serum. (a) For the presence of complete anti-Rh agglutinins: When they were available group O red cells of Rh_0 (cDe), Rh′ (Cde), Rh″ (cdE), and Rh-negative (cde) subgroups were used. More often, however, only Rh_1 (CDe), Rh_2 (cDE), and Rh-negative (cde) cells were available. Artificial Rh′ (Cde) and Rh″ (cdE) cells were prepared by mixing equal parts of weak suspensions of Rh_1 (CDe), and Rh_2 (cDE) cells with serum containing the "incomplete" antibody (Δ') in high titre. The nature of an anti-Rh agglutinin could then be determined as shown in Table 2. The method of performing the tests and of examining the mixtures is as described earlier (Walsh, 1945).

TABLE 2.

Reactions of sera against red cells of various Rh subgroups.

Type of Agglutinin.	rh rh (cde)	Rh_1 (CDe)	Rh_2 (cDE)	Artificial Rh′ (Cde)	Artificial Rh″ (cdE)
Anti-Rh_0 Δ	—	+	+	—	—
Anti-Rh′ Γ	—	+	—	+	—
Anti-Rh″ H	—	—	+	—	+
Anti-Rh_0′ $\Delta\Gamma$	—	+	+	+	—
Anti-Rh_0″ ΔH	—	+	+	—	+

(b) For the presence of the incomplete antibody (Δ'):

(1) Wiener's original test (Wiener, 1944) can be used either as a qualitative or as a quantitative test. The qualitative test was performed by adding a drop of the unknown serum to a drop of a weak suspension of Rh_0 (cDe) cells. The mixture was incubated for 30 minutes at 37° C. A drop of serum containing the anti-Rh_0 (Δ) agglutinin was added, the mixture was incubated for a further 90 minutes and then examined microscopically. Absence of agglutination indicated the presence of an incomplete antibody. A control was set up using normal serum (not immunized) as well as the unknown serum. If the agglutination in the unknown was less marked than that in the control, the incomplete antibody was presumed to be present in very small concentration.

The quantitative test was performed by titrating the unknown serum in serial dilutions against a suspension of Rh_0 (cDe) red cells, incubating the mixtures at 37° C. for 30 minutes and then adding to each tube a drop of a serum known to contain the Rh_0 (Δ) agglutinin. After incubation for a further 90 minutes the mixtures were examined microscopically. The titre of the incomplete antibody (Δ') was taken to be the final dilution of the unknown serum (after addition of the red cells) in which there was no agglutination. The titre has been found to be independent of the titre of the anti-Rh_0 (Δ) serum used (Sanger, 1946).

This test will be referred to as the "Saline test" for the blocking antibody.

(2) *Albumin test.* This test may also be either qualitative or quantitative. Solutions of albumin (25 p.c.), prepared at the Department of Physical Chemistry, Harvard University Medical School, were available and satisfactory. The albumin solution was used both for suspending the red cells and for serially diluting the unknown serum. The titration was performed in the usual manner. Saline was, however, not added at any stage to any of the mixtures. No great difficulty was experienced in determining the end-point.

(3) *The "Conglutination" test.* This test was performed as described by Wiener (1945). It was found unsatisfactory because rouleaux formation was almost invariably present and a satisfactory end-point could not be obtained on microscopic examination. The authors believe that microscopic examination of the red cells is essential, having found naked eye examinations fallacious, especially for quantitative measurements.

(4) *Diamond's test.* The test described by Diamond and Abelson (1945), in which a thick suspension of the cells is mixed with the unknown serum, was used. It also was found to be unsatisfactory, firstly, because it is not a quantitative test, and secondly, because rouleaux were commonly present and could not be distinguished from minor degrees of true agglutination.

(5) *The "C.M.R." test.* Serum from rabbits immunized against human serum was used for the test[1] in a dilution of 1 in 40 (this dilution giving strong clumping of positive controls). Both qualitative and quantitative tests were performed. In the qualitative test the unknown serum was mixed with the test cells of known Rh subgroups, usually Rh_0 (cDe) cells. After the mixture was incubated at 37° C. for 30 minutes the cells were washed three times with normal saline to remove all traces of human serum except that which was attached to the surface of the red cell as a result of a specific antigen-antibody reaction. A drop of the diluted anti-human serum was then added to the washed red cells and this mixture incubated for 30 minutes. The red cells were examined microscopically for the presenec or absence of agglutination. A serum known to contain an incomplete antibody (Δ') and a serum known to lack the antibody were always used as controls. The quantitative test was performed by adding a suspension of Rh_0 (cDe) cells to serial dilutions of the unknown serum, incubating the mixtures at 37° C. for 30 minutes and then washing the red cells in each tube free of surrounding serum. Anti-human serum, diluted 1 in 40, was added to the washed red cells in each tube and the mixtures incubated and examined as in the qualitative test. The original dilution of the unknown serum in the last tube (before the cells were washed) to show agglutination was taken to be the antibody titre.

RESULTS.

Twenty-five sera, obtained from mothers of babies suffering from Haemolytic Disease of the Newborn, did not contain any variety of complete anti-Rh agglutinins when tested in saline. Quantitative albumin, saline blocking tests, and "C.M.R." tests were performed on twenty-one of these sera with the results shown in Table 3. The other four sera (Cases 22 to 25) all gave positive albumin and qualitative saline blocking tests, but were not tested by the "C.M.R." test.

A further thirteen sera contained complete anti-Rh agglutinins as shown in Table 4. In two instances (Cases 26 and 29) the agglutinin was not the anti-Rh_0 (Δ) variety, but there was an incomplete antibody of Rh_0 (Δ') specificity, as detected by the "Saline test". In another instance (Case 27) the serum contained an anti-Rh′ (Γ) agglutinin, but the saline test was negative for incomplete antibody (Δ'). However, the albumin test gave a titre of 1 in 32 against Rh_0 (cDe) cells. In Case 28 an anti-Rh_0 (Δ) agglutinin gave a titre of 1 in 8 in

[1] This serum was kindly supplied by the N.S.W. Department of Public Health.

TABLE 3.

Results of various tests on twenty-one sera.

Case No.	Albumin test.	Saline test.	"C.M.R." test.	Case No.	Albumin test.	Saline test.	"C.M.R. test.
1	1/128	1/8	+	11	1/32	—	+
2	1/128	1/4	+	12	1/32	—	+
3	1/128	1/2	+	13	1/16	1/8	+
4	1/128	—	+	14	1/16	1/8	+
5	1/128	—	+	15	1/16	1/4	+
6	1/32	1/4	+	16	1/16	—	+
7	1/32	1/4	+	17	1/8	—	+
8	1/32	1/2	+	18	1/8	—	+
9	1/32	—	+	19	1/8	—	+
10	1/32	—	+	20	1/4	—	+
				21	1/2	—	+

+ = agglutination.

saline, but of 1 in 128 with the albumin test. A prozone phenomenon was obtained with the saline titration, agglutination being absent with the serum diluted 1 in 2 but present when diluted 1 in 4 and 1 in 8. Further addition of anti-Rh_0 (Δ) agglutinins to the 1 in 2 serum dilution failed to produce agglutination, indicating the presence of an incomplete antibody (Δ'). Moreover, red cells of the Rh_1 (CDe) and Rh_2 (cDE) subgroups gave a positive "C.M.R." test after having been mixed with this serum diluted 1 in 32. In seven other instances (Cases 32 to 38) the agglutinin titres in saline and in albumin were identical.

TABLE 4.

Results of various tests on thirteen sera containing anti-Rh agglutinins.

Case No.	Type of agglutinin.	Titre of agglutinin.	Albumin test against Rh_0 cells (cDe)	Saline test.	"C.M.R." test.
26	Anti-Rh" (H)	1/32	1/32	1/4	+
27	Anti-Rh' (Γ)	1/16	1/32	—	+
28	Anti-Rh_0 (Δ)	1/8	1/128	1/2	+
29	Anti-Rh (Γ')	1/4	1/64	+	0
30	Anti-Rh_0' ($\Delta\Gamma$)	1/128	1/256	1/4	+
31	Not identified	1/2	1/16	—	+
32	Anti-Rh_0 (Δ)	1/16	1/16	—	0
33	Anti-Rh_0 (Δ)	1/16	1/16	—	0
34	Not identified	1/64	1/64	—	0
35	Not identified	1/8	1/8	—	0
36	Anti-Rh_0 (Δ)	1/2	1/2	—	0
37	Anti-Rh_0 (Δ)	1/8	1/8	—	0
38	Anti-Rh_0 (Δ)	1/256	1/256	—	0

0 = test not performed.

Case 30 is of particular interest. Agglutination in the saline titration of the serum against Rh_0 (cDe) cells was absent in the 1 in 2 and 1 in 4 dilutions, weak in the 1 in 8 dilution, reached maximum intensity in the 1 in 32 dilution, and was present up to 1 in 128.

Case 36 is an instance of the development of an anti-Rh_0 (Δ) agglutinin in a patient of the subgroup Rh' (Cde). Until the Rh subgroup of the patient was determined the presence of an anti-Rh agglutinin in an Rh-positive patient was confusing.

Tests were performed on several samples of serum obtained from a patient (Case 39) and the results demonstrate the probable development *in vitro* of an incomplete antibody (Δ').

On 18/1/46, anti-Rh_0' ($\Delta\Gamma$) agglutinins were demonstrated with a titre of 1 in 128 against Rh_1 (CDe) cells. The albumin test gave the same titre. Similar results were obtained on 9/2/46 and on 27/2/46. A quantity of oxalated blood was collected from the patient on 5/3/46 and, after the plasma had been clotted, the serum was removed. This serum was filtered through "Hormann Ekwip D9" filter pads and placed in ampoules. On 18/3/46 the serum in one of these ampoules was further tested. It contained anti-Rh' (Γ) agglutinins with a titre of 1 in 32 against Rh_1 (CDe) cells, but no anti-Rh_0 (Δ) agglutinins. The "Saline Test" for incomplete antibody (Δ') gave a titre of 1 in 16. The albumin test against Rh_0 (cDe) cells was positive with a titre of 1 in 64, and the "C.M.R." test gave a positive result against Rh_0 (cDe) cells. A further sample of

serum was obtained from the patient on 25/3/46 and was tested immediately after collection. The agglutinin was again of the anti-Rh_0' ($\Delta\Gamma$) variety, with a titre of 1 in 128 against Rh_1 (CDe) cells. The albumin titre was 1 in 256 against both Rh_1 (CDe) and Rh_2 (cDE) cells. In the saline titration, agglutination with the serum diluted 1 in 2 was weaker than that in the following tubes, indicating the presence of a very weak incomplete antibody (Δ'). This last sample of serum was obtained and tested to exclude, with reasonable certainty, the possibility that the strong, incomplete antibody (Δ') demonstrated on 18/3/46 may have developed *in vivo*.

Case 40 was that of an Rh-positive patient. The reactions given by this serum are shown in Table 5. The reactions obtained against albumin suspensions of the red cells of a large number of individuals were consistent with those of the anti-St (s) agglutinin.

TABLE 5.

Results of Case No. 40.

Test cells.	Saline titre.	Albumin titre.
rh (cde)	1/8	1/16
Rh_0 (cDe)	—	1/16
Rh_1 (CDe)	—	—
Rh_2 (cDE)	1/8	1/16
Rh′ (Cde)	—	—
Rh″ (cdE)	1/8	1/16

The result of the "C.M.R." test on the serum of Case 28 suggested the performance of comparative quantitative "C.M.R." and albumin tests on serum known to possess an incomplete antibody (Δ') as shown by the "Saline test". It was found that the titre of the quantitative "C.M.R." test was approximately four times that of the albumin test. The results obtained on seven such sera are shown in Table 6.

TABLE 6.

A comparison of the albumin and quantitative "C.M.R." tests.

Serum.	Albumin titre.	"C.M.R." test. Titre.
Mrs. W.	1/64	1/256
Mrs. B.	1/32	1/128
Mrs. H.	1/64	1/256
Mrs. C.	1/16	1/64
Mrs. Hu.	1/16	1/128
Mrs. M.	1/8	1/32
Mrs. A.	1/16	1/128

The relative affinities of the anti-Rh_0 (Δ) agglutinin and the incomplete antibody (Δ') for Rh_0 (cDe) red cells was investigated by mixing a serum containing the former with different amounts of a serum containing the latter and allowing the mixture to act on Rh_0 (cDe) cells. The titre of the anti-Rh_0 (Δ) agglutinin was 1 in 64 and the albumin titre of the incomplete antibody (Δ') 1 in 64. One drop of the mixed sera was added to one drop of a saline suspension of the red cells in each instance. The proportion of the two sera used and the results of microscopic examination are shown in Table 7. In this experiment the two sera were mixed together for 30 minutes before being added to the cells. The period of preliminary mixing is important, because in a further experiment a mixture of 3 parts of a serum containing anti-Rh_0 (Δ) agglutinin and 1 part of a serum containing the incomplete antibody (Δ') produced agglutination when added to Rh_0 (cDe) cells immediately, 5 minutes and 10 minutes after mixing. It failed to do so, however, 40 minutes after the two sera were mixed together.

TABLE 7.

Relative affinities of the anti-Rh_0 agglutinins and the incomplete antibody for Rh_0 red cells.

Proportion of anti-Rh_0 (Δ) serum to incomplete antibody serum (Δ')	5/1	3/1	2/1	1/1	1/2	1/3	1/5
Results of reactions	+	+	+	+	±	—	—

A prozone phenomenon against Rh_0 (cDe) red cells was observed with the sera of both Cases 28 and 30. A further serum (Mrs. T.) gave a titre of 1 in 64 in saline against Rh_0

(cDe) cells, but there was no agglutination with serum diluted less than 1/16. The red cells which had been exposed to the serum diluted 1 in 2, 1 in 4, and 1 in 8 were washed free of serum and then mixed with anti-human serum. In all three instances agglutination was observed, indicating that protein had been attached to the surface of the red cells even though agglutination was not produced.

DISCUSSION.

A widely accepted concept of antigen-antibody reaction is that proposed by Marrack (1938). This is based on the formation of a lattice by multivalent antibodies uniting antigen molecules. According to this theory the anti-Rh agglutinin is presumably bivalent or multivalent and when the combining sites are attached to the specific red cell receptors, agglutination is produced. These agglutinins are referred to as complete agglutinins and agglutinate red cells suspended in saline.

A concept of the mechanism of the incomplete antibody is that it is univalent. It is unable to serve as a link between red cells but effectively combines with the receptors on the red cells, thus preventing agglutination by bivalent or complete antibodies. These concepts are illustrated in a diagram (Fig. 1) modified from Wiener (1945b).

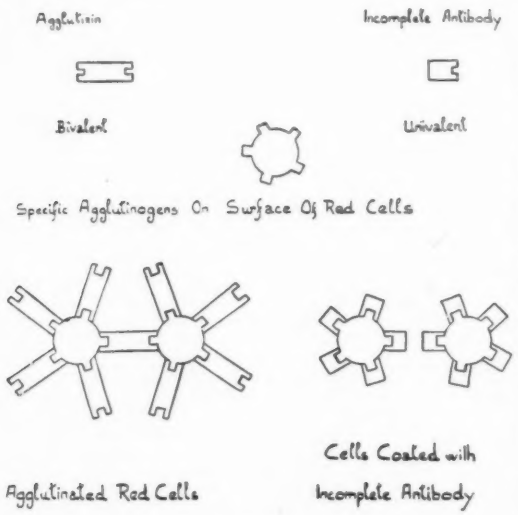

Fig. 1.

The discussion which follows is based on the above hypothesis. Consequently, the terms univalent antibody and incomplete antibody are synonomous.

The "Saline Test" is the only direct test for detecting the presence of the incomplete antibody in serum. It is believed that the albumin test also indicates its presence, because there is a good correlation between the results of this test and the clinical condition of the infant. It can, however, be seen from Tables 3 and 4 that there is no quantitative correlation between the titre of the albumin tests and the results of the "Saline Test". Nevertheless, the albumin test was never negative if the saline test was positive.

It has been pointed out (Abramson, Moyer, Gorin, 1942) that the red blood corpuscle is somewhat unique because its surface does not absorb protein unless there has been a specific union of antigen and antibody on the surface. The "C.M.R." test, which indicates the presence of any attached protein, and therefore is evidence of a specific union, lends support to the belief that the albumin test does indicate the presence of an incomplete antibody. The qualitative "C.M.R." test was positive whenever the albumin test was positive, and there was a good correlation between the titre of the albumin test and that of the quantitative "C.M.R." test in the sera compared. It may be presumed on this evidence that the albumin test does indicate a specific union of an antibody with an antigen on the surface of the red cell. The higher titres of the quantitative "C.M.R." test, however, suggest that it is a much more sensitive test than is the albumin test.

It is possible that the incomplete antibody is a manifestation *in vitro* of a slightly altered bivalent or complete antibody. The effects *in vivo* of the incomplete antibody are identical with those of the complete antibody, in respect to the causation of both Haemolytic Disease of the Newborn and transfusion reactions. Further, when Rh-positive cells are suspended in serum, as in Wiener's "Conglutination" test, or in albumin, agglutination is produced by the in-

complete antibody. On this assumption the manifestations of the incomplete antibody are similar to those of a complete antibody in any environment which inhibits the dissociation of the altered complete antibody. Such environment could be provided in the albumin and "Conglutination" tests, whereas on dilution with saline dissociation occurs and the blocking effect is produced. If this is not correct, the mechanism by which the incomplete antibody produces agglutination when the red cells are suspended in albumin is obscure. There is not sufficient evidence at the present time to substantiate any suggestion that the albumin molecule is able to unite two univalent antibodies.

The apparent development *in vitro* of the incomplete antibody (Δ') in the serum of Case 39 may be due to the dissociation of bivalent or multivalent agglutinating antibodies, which is a mechanism suggested by Tyler (1945). On the other hand, it may be due to the destruction of the complete antibody, and the unmasking of the incomplete antibody. The latter theory is proposed by Diamond and Abelson (quoted by Boyd, 1946) and received support from the work of Boyd (1946), who was unable to produce incomplete antibodies by photo-oxidation of a serum containing the anti-Rh_0 (Δ) agglutinin. However, only a few sera containing anti-Rh_0 (Δ) agglutinins and treated in a manner similar to that of Case 39 ever develop an incomplete antibody (Δ'). This fact and its very rapid development in Case 39 support the hypothesis of dissociation rather than that of destruction. It is not known what factors are responsible for the development *in vitro* of an incomplete antibody in a serum which previously contained a complete antibody. If the hypothesis that the univalent antibody results from the dissociation of the bivalent antibody is correct, it is necessary to explain why some sera dissociate immediately after collection when others do not do so for some time, or not at all. When serum containing incomplete antibody was mixed with serum containing complete antibody the results obtained varied with the time elapsing between the actual mixing and the addition of red cells. This may indicate the partial dissociation of the complete antibody by some factor in the serum which contains the incomplete antibody. This point will be investigated in the future. Some sera immediately after collection contain the incomplete antibody (Δ') when tested by the "Saline Test". Agglutination, however, is produced in the albumin and Conglutination tests. It would seem that the dissociation of the complete antibody in these instances is produced by the dilution of the serum with saline.

An incomplete antibody (Δ') may be present in a serum which also contains an anti-Rh_0 (Δ) agglutinin. If the agglutinin (Δ) is stronger than the incomplete antibody (Δ') agglutination will result. On the other hand, if the incomplete antibody (Δ') is stronger than the anti-Rh_0 (Δ) agglutinin, the "blocking" phenomenon will be observed. The production of an artificial anti-Rh′ (Γ) serum from an anti-Rh_0′ ($\Delta\Gamma$) serum by mixing it with a serum containing a strong incomplete antibody (Δ') is based on this finding. The success of this procedure is dependent on the use of a serum with the incomplete antibody (Δ') in high titre. When both complete and incomplete antibodies are present in a serum, the titres obtained by the saline and albumin tests will vary according to the relative concentration of the two antibodies. Both titres would presumably be identical if either the incomplete antibody is absent or if its titre is less than that of the complete antibody. If this assumption is correct then the fact that the albumin titre is not higher than the saline titre, would not necessarily indicate absence of the incomplete antibody. On the other hand, if the albumin titre is higher than the saline titre, an incomplete antibody is presumed to be present also.

A prozone phenomenon is often observed, especially when there is a large difference between the saline and albumin titres. It was demonstrated that the red cells, which are not agglutinated by the lower dilutions of a serum showing the prozone phenomenon, nevertheless, do absorb some fraction of the serum protein as a result of a specific antigen-antibody reaction. In view of this fact, and because a prozone phenomenon is observed only with those sera containing the incomplete antibody in high titre, it is suggested that the incomplete antibody may be responsible for the phenomenon. It is, however, difficult to explain the phenomenon on this basis with any precision. In a serum which contains both, the proportion of an incomplete antibody (Δ') and an anti-Rh_0 (Δ) agglutinin will be independent of the dilution of the serum. If the undiluted serum fails to agglutinate Rh_0 (cDe) red cells it is presumed that the receptors on the red cell surfaces are all coated with the more abundant univalent molecules before the bivalent agglutinin molecules are able to produce agglutination. When the serum is diluted with saline the agglutinins are able to attach themselves to the cell receptors and produce agglutination of the cells, even though the bivalent agglutinin molecules are still outnumbered by the univalent antibody molcules. The ability of diluted serum to bring about agglutination may be due to the fixation of all the univalent antibody molecules without the utilization of all the receptors on the red cell surfaces. The less numerous bivalent molecules are then able to become attached to the remaining receptors and produce agglutination. There is, however, no evidence to support such a quantitative theory which also implies a relatively greater affinity of the incomplete antibody for the cell receptors. In contrast it is possible that in the diluted serum the complete agglutinin is more readily attached to the cell receptors than is the incomplete antibody, merely because of the environmental changes. The problem is receiving further attention.

The reactions given by the serum of Case 40, and shown in Table 5, are somewhat difficult to explain. They could be interpreted as evidence of the presence of an incomplete anti-St (8) agglutinin, especially in relation to the reactions with Rh_0 (cDe) red cells. But no evidence could be obtained with rabbit anti-human serum that Rh_0 (cDe) red cells suspended in saline and exposed to the serum were coated with a specific antibody. There is, therefore, no direct evidence in this instance that the albumin test indicates the presence of an incomplete anti-St (8) antibody.

The saline "blocking" test served a very useful purpose in that it drew attention to the existence of the incomplete antibody. It is, however, too insensitive to be employed as the routine test for the detection of iso-immunization. The albumin test is accurate and fairly easy to perform. If supplies of albumin are available it is probably the test of choice. The "C.M.R." test, although a more sensitive and, if properly controlled, accurate test, is more difficult to perform because the cells must be carefully washed. As the originators have suggested, it will be useful in the investigation of other antigen antibody reactions. In our hands Wiener's conglutination and Diamond's thick-cell suspension tests have not been found suitable for routine use.

SUMMARY.

The various tests used to detect iso-immunization against the Rh factor are compared in the serum from 38 individuals.

Evidence is produced to indicate that the albumin test signifies the presence of an incomplete antibody.

The incomplete antibody possibly exists *in vivo* as slightly altered complete antibody and acquires its "blocking" properties *in vitro*.

The reactions of an anti-St (8) agglutinin are described and discussed.

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SOME FACTORS INFLUENCING THE VIRULENCE OF *HAEMOPHILUS PERTUSSIS* PHASE I

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Pertussis infection is an important disease of childhood, for which—apart from preliminary experiments with Streptomycin in mice (Hegarty and his colleagues, 1945)—no specific chemotherapy exists. Any preventive measures which may tend to mitigate the severity of this disease are therefore of importance in the field of human medicine.

Since the demonstration of the value of using antigenically smooth strains of *H. pertussis* for vaccine production, namely Phase I (Leslie and Gardner, 1931), confidence in immunization has been partly restored. Most workers report a significant reduction in incidence as well as in the severity of the attack in actively immunized children (Sauer, 1933a, 1933b, 1935, 1939, 1942; Kendrick and Eldering, 1936; Howell, 1938; Schermerhorn, 1938; Bell, 1941; Daughtry-Denmark, 1942; Perkins and colleagues, 1942; and Dungal and colleagues, 1944). Nevertheless, other workers (Siegel and Goldberger, 1937; Doull and his colleagues, 1939; and McFarlan and his colleagues, 1943, 1945) have reported unfavourably on vaccination.

These rather conflicting findings are not easy to reconcile. One explanation which might be advanced is that the vaccines used by different groups of workers possessed different immunizing properties. This explanation implies that phase I, *H. pertussis*, in spite of its apparent homogeneity when tested by agglutination may vary in the degree of protection it confers as a vaccine.

Despite the prevalent belief that under certain fairly rigid conditions of maintenance on artificial medium *H. pertussis*, phase I does not undergo significant variation in virulence and antigenicity (Burnet and Timmins, 1937; North and his colleagues, 1939; Wood, 1940; and others), the literature reveals that several workers, including Leslie and Gardner (1931), Lawson (1939) and Flosdorf and Kimball (1940a, 1940b) were not convinced that this was so. Leslie and Gardner note, without comment, that phase I strains alter serologically within a few months of isolation, while Lawson and Flosdorf and Kimball respectively employed frequent mouse passage and vacuum drying to prevent unexplained fluctuations in virulence of a single infecting strain of phase I, *H. pertussis*.

It was evident that at least some of the failures of the intra-nasal infection technique of Burnet and Timmins (1937) were due to the use of cultures which were either old, or of which the physiological age was not specified. North and his co-workers (1939, 1941, 1942) produced uniformly fatal infections by this method with doses as low as 50 million organisms, while others, such as Eldering (1942), Miller and Silverberg (1939) and Silverthorne (1938) failed to kill all controls, even when doses as high as 12,000 million organisms were instilled intranasally.

The experiments recorded here represent an attempt to examine more closely some characters, other than antigenic structure, which may influence the immunizing potency of vaccines prepared with this organism. To this end the

1 This work was assisted by a grant from the National Health and Medical Research Council.

property of virulence for mice of *H. pertussis* was investigated. The results showed that striking differences occur in the virulence of cultures which would otherwise be regarded as identical antigenically and that this change in virulence was associated with the physiological age of the culture. Less dramatic changes in virulence could be demonstrated under the influence of the medium employed, being enhanced by growth in the developing egg and reduced by serial subculture on Bordet-Gengou (B-G) medium. Furthermore, these variations have been found to correlate closely with mouse immunizing potency. These latter results are being prepared for publication.

MATERIALS AND METHODS.

Standardization of variables in these experiments was aimed at wherever possible and statistical analysis employed to interpret the results.

Origin and Maintenance of H. pertussis.

One strain, picked from a cough plate in the catarrhal stage of pertussis in a child and conforming to Leslie and Gardner's definition of phase I was used throughout. Its original antigenicity was ensured by vacuum drying the culture, after its initial identification, in a large number of ampoules. At the same time, for comparative purposes, serial subcultures were maintained on Bordet-Gengou medium containing 30 p.c. of fresh citrated horse blood. To commence any given experiment an ampoule of the original culture was sown on to a B-G plate and incubated at 37° C. After visible growth appeared, usually within 60 hours, subcultures were made for the experiment proper. The time of this subculture is taken as zero in computing the physiological age. Hence for all subsequent experiments it can be claimed that the organisms were subcultured only four times after isolation from the human body.

The Physiological Age of Cultures.

The decision to select definite incubation times at which cultures would be tested for virulence was dictated by the failure of other workers to consider this factor and the diversity of their subsequent results. On B-G medium the yield from a 24-hour culture was approximately one-half that of a 48-hour culture, and one-third that of a 72-hour culture, assuming that the same inoculum was used in each instance. Reduction of the incubation time below 24 hours failed to yield workable quantities.

The failure to secure satisfactory growth of fully virulent organisms in fluid medium except in the presence of blood or haemin introduced difficulties in estimating the number of organisms per ml. of fluid which could not at the time be overcome.

Very young cultures, which might reasonably be expected to possess an even greater virulence (Bazeley, 1943), were therefore not tested. Suspensions for virulence tests were prepared by washing off the growth at appropriate intervals with warm, buffered broth[2] and standardizing, after coarse filtration through cotton wool, to Wellcome (Brown's) No. 9 opacity tube. This is equivalent on their figures to approximately 10,000 million organisms per ml. in terms of *H. influenzae*. This figure has been accepted here as a sufficiently accurate estimate of the numbers of *H. pertussis* present in a suspension, because, the experiments being purely comparative, actual numbers are not very important. The accuracy of the opacity of the suspension was checked by dilution 1 in 4·5 to correspond with tube No. 2, at which turbidity comparisons are less subject to visual error.

Cultivation in the Developing Chick Embryo.

In view of the relative ease with which *H. pertussis* adapts its nutritional requirements to increasingly unfavourable media, undergoing gross variation in the process, experiments were performed to determine whether growth in the presence of living cells might favour the maintenance of those properties associated with virulence to a greater extent than a non-living medium.

A series of early observations on routes of inoculation and the use of progressively older embryos yielded the following information:

(i) Of the various routes employed, namely, into either the allantoic cavity, the amniotic cavity or the yolk sac, the former yields the highest concentration of organisms in the allantoic and amniotic fluids and is the most readily performed.

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2 *Buffered broth*: This medium, which has been found suitable for the growth of fully virulent *H. pertussis* in fluid medium, consists of B-G medium, without agar, to which are added buffer salts to stabilize any tendency to a metabolic swing in pH.

The buffer mixture contains 0·0235 p.c. of Na_2HPO_4 and 0·0115 p.c. of $NaH_2PO_4 \cdot 2H_2O$. Sterilization is by Seitz filtration. For growing *H. pertussis* 10 p.c. of laked horse blood is added, but as a non-toxic diluent buffered broth is used alone.

(ii) The optimal embryo age is six or seven days. With increasing age the growth of *H. pertussis* is more strongly resisted by the embryo, so that from the twelfth day onward infection is often overcome. Such eggs not infrequently hatched chicks with no clinical or bacteriological evidence of infection.

(iii) *H. pertussis* kills six- or seven-day embryos in 48 to 72 hours but, as would be expected, continues to multiply for some time after the death of the embryo, and is found in high concentration in the egg fluids.

The technique adopted was slightly modified from that of Burnet (1936), maintaining a high incubator humidity to reduce evaporation of embryo fluids. A spot on the shell is marked with pencil close to the embryo, but free from major vessels, where the allantoic cavity is well formed at this stage of incubation. At the same time the airspace is outlined. Using a dental drill the sterilized shell is pierced over the airspace and at the inoculation site. The latter is covered with an antiseptic pad until the inoculum is introduced. The inoculum, consisting of approximately 0·2 ml. of a heavy suspension of 24-hour culture from B-G medium, is introduced with a fine Pasteur pipette, a little allantoic fluid having been previously drawn up to ensure that the chorio-allantois has been pierced. The hole into the airspace allows internal pressure to equalize and prevents overflow of the inoculum on to the shell surface. The openings are then sealed with sterile paraffin and the egg incubated at 37° C. in a humid atmosphere for a period appropriate to the experiment.

The growth is harvested by aseptically removing the shell overlying the airspace and slitting the shell membrane and underlying chorio-allantois and amnion. From 4 to 6 ml. of syrupy, yellowish or straw-coloured fluid is sucked off, avoiding the viscid contents of the albumin sac. Any abnormal odour is usually an indication of contamination.

Figs. showing growth of *H. pertussis* in the developing chick embryo, and the recovery of the organisms from the allantoic fluid.

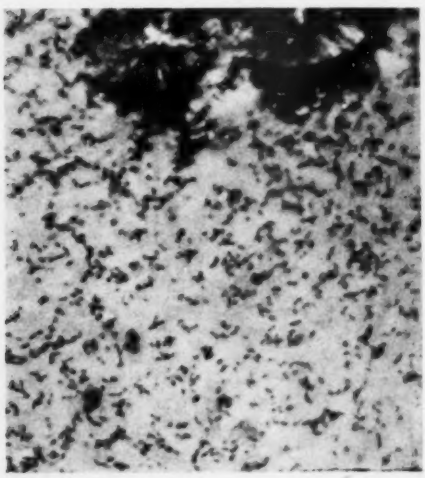

Fig. 1. *H. pertussis.* 48 hours at 37° C. in a 7-day embryo. Direct smear of allantoic fluid. Dilute carbol fuchsin 2 minutes. (X900).

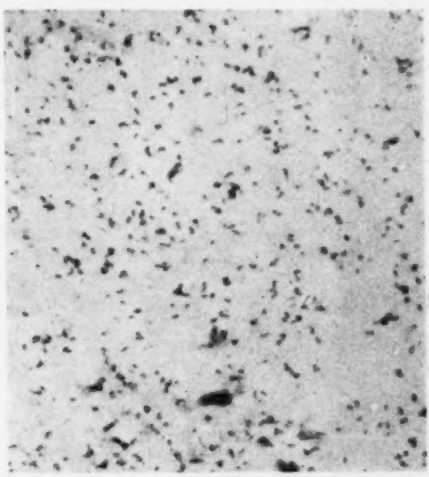

Fig. 2. Centrifuge deposit resuspended in saline to 10,000 million *H. pertussis* per ml. Little residual protein. (X900).

Thin smears dried in air, cleared with spirit, and stained for at least two minutes with dilute carbol fuchsin—either as a simple stain, or as a counter-stain in Gram's method—show a high concentration of organisms (Fig. 1). Removal of the bulk of the egg protein requires dilution of the fluid in 10 to 15 volumes of buffered broth and mechanical shaking with beads for twenty minutes. When filtered through wool and centrifuged at 4,000 r.p.m. for 60–70 minutes, the organisms are deposited as a viscid greyish white mass and the bulk of the egg protein remains in the supernatant fluid. On gentle trituration in a mortar with 2 ml. of buffered broth and standing for about thirty seconds the milky supernatant may be pipetted off, diluted to approximately 20,000 million organisms per ml. and filtered through cotton wool (Fig. 2). This, standardized to Brown's No. 9 opacity tube, provides a suitable suspension,

consisting mainly of *H. pertussis*, for virulence tests. The average yield from a 48-hour egg culture is about 5 ml., containing 10,000 million organisms per ml. It will be noted that no allowance has been made in standardizing suspensions from egg culture for the opacity of residual egg protein. This means that the actual numbers are lower than the counts indicate, thus weighting the results, in a comparison of egg and B-G culture, against the egg preparation.

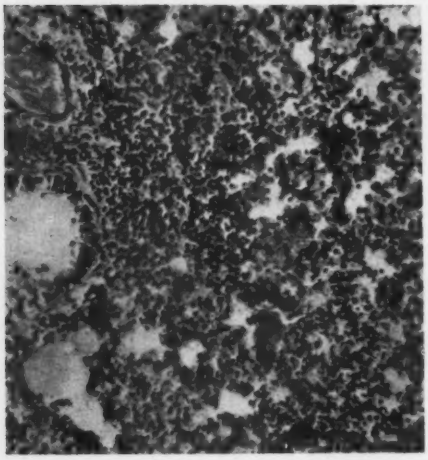

Fig. 3. Death within 24 hours of inoculation. (X150).

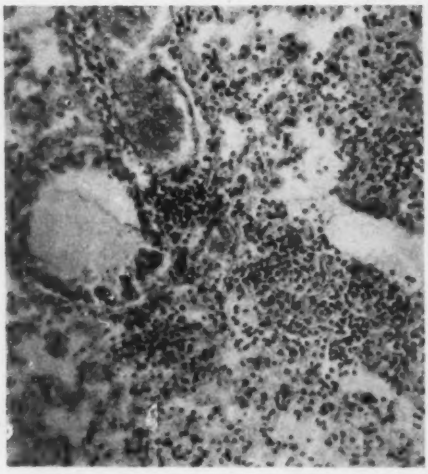

Fig. 4. Death in two days. (X150).

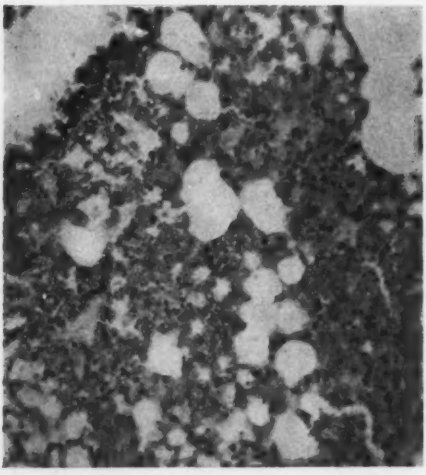

Fig. 5. Death in three days. (X150).

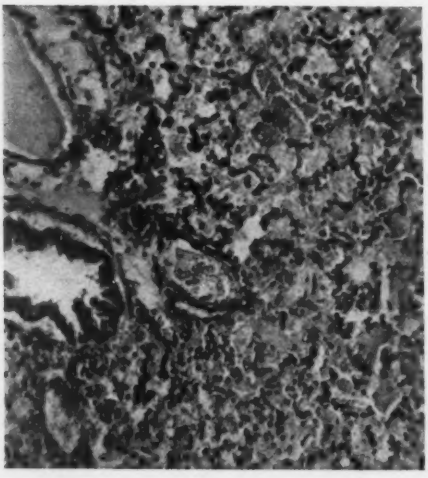

Fig. 6. Death in three days. (X150).

Experimental Animals.

Although it would seem that little importance has been attributed to the age of the experimental animals, it was decided here to employ white mice three weeks of age of the same closely-bred strain, maintained on a uniform diet. There was no history of intercurrent infec-

tion in these animals, but during the course of the experiments each mouse that died was examined to ensure that the experimental procedures were responsible for its death.

Method of Infection and Assessment of Degree.

Following the method of Burnet and Timmins (1937) the test suspensions were instilled intranasally under light ether-chloroform anaesthesia. Serial dilutions in buffered broth were used in each experiment. As far as possible the time elapsing between removal of the plates from the incubator and the instillation of the appropriate dilution into mice was kept constant. In no case did it exceed one hour. The dose volume, 0·05 ml., was measured as three drops from a 60-drop pipette at the rate of approximately one drop per second, delivered from a distance of 0·5 cm. above the nostrils. The mouse was held in a vertical position during the process, and any mouse failing to inhale the dose evenly was discarded, as also was any which failed to recover satisfactorily from the anaesthetic.

This method was adopted in preference to the intraperitoneal route because, under specified conditions and with a relatively small dose compared with that required for a fatal intraperitoneal injection, it produces a uniformly fatal specific pneumonia which is independent of the endotoxin content of the inoculum. This will be discussed in detail at a later stage.

At post-mortem examination the lungs present a characteristic macroscopic and microscopic pathology (Burnet and Timmins, 1937; Bradford, 1938). Those animals dying in less than 24 hours show a severe haemorrhagic oedema, cloudy swelling of the bronchial epithelium and early polymorphonuclear diapedesis, with little or no fibrin in the alveoli. Where the

TABLE 1.

The influence of physiological age on the virulence of B-G cultured H. pertussis.

Series.	Inoculum.	No. of mice.	No. of organisms.	Time elapsing before death. Hours.	Survivors 21 days.	Avr. survival time.
1.	24-hr. B-G. culture	5	500 miln.	17 17 17 17 17	0/5	17 hrs.
		5	250 miln.	17 17 17 17 18	0/5	17 hrs.
		5	100 miln.	17 17 17 17 17	0/5	17 hrs.
		10	50 miln.	18 18 18 21 21 22 26 28 30 30	0/10	23 hrs.
				Days.		
		5	25 miln.	5 6 7 8 9	0/5	7 days
		5	10 miln.	7 8 8 9 12	0/5	8·8 days
		10	5 miln.	4 4 4 5 7 8 9 14 14 17	0/10	8·6 days
		10	2·5 miln.	5 5 5 6 7 7 8 8 18 19	0/10	8·8 days
				Days.		
2.	48-hr. B-G. culture	5	500 miln.	2 2 4 6 7	0/5	4·2 days
		5	250 miln.	2 5 7 8 8	0/5	6·0 days
		5	100 miln.	6 9 11 11 S*	1/5	11·8 days
		5	50 miln.	5 8 10 14 S	1/5	11·8 days
		5	25 miln.	9 10 11 13 S	1/15	13·0 days
		10	10 miln.	5 5 6 8 9 12 13 S S S	3/10	12·4 days
		10	5 miln.	7 10 10 10 12 12 13 14 S S	2/10	13·2 days
		5	2·5 miln.	6 10 12 12 S	1/5	12·4 days
				Days.		
3.	72-hr. B.G. culture	5	500 miln.	10 14 16 S S	2/5	16·8 days
		5	250 miln.	10 18 S S S	3/5	18·8 days
		5	100 miln.	14 S S S S	4/5	20·4 days
		5	50 miln.	14 S S S S	4/5	20·4 days
		5	25 miln.	S S S S S	5/5	22 days
		5	10 miln.	S S S S S	5/5	22 days
		5	5 miln.	S S S S S	5/5	22 days
		5	2·5 miln.	S S S S S	5/5	22 days
4	Buffered Broth	5	Nil	S S S S S	5/5	22 days

*S = Survived 21 days. For the purpose of assessing the average survival time these animals are recorded as having survived 22 days (see Fig. 7).

survival time exceeds 24 hours, intra-alveolar haemorrhage and congestion are less evident and the pathology becomes not unlike that of early hepatisation in human lobar pneumonia. Emphysema and fibrin exudation into the alveoli become more pronounced and lymphocytes, plasma cells and macrophages are found in increasing numbers from the third day onwards. Figs. 3–6 show the gross histological changes in mice dying at different intervals after infection. From the lesions fully virulent *H. pertussis* is always recoverable, frequently in almost pure culture.

Interpretation.

Results of virulence experiments are recorded in terms of percentage deaths and average survival times. For computing average survival times it was considered permissible, merely as a matter of convenience, to record all animals alive at the twenty-first day of the experiment as having survived for 22 days, regardless of the fact that many would have lived much longer or even have recovered completely. The average survival times, therefore, favour the less virulent cultures.

RESULTS.

The Influence of Physiological Age on the Virulence of Artificially Cultured H. pertussis.

A series of plates from the same batch of medium sown from the same ampoule recovery plate were harvested at the appropriate intervals and standardized to 10,000 million organisms per ml. as described above. The three series of mice were treated at intervals of 24 hours, so that the 72-hour series was on the average two days older than the 24-hour series. This difference in age is not considered significant. Table 1 summarizes the experiment.

It will be seen in the series receiving 24-hour culture that all the dose ranges from 500 million down to 50 million were fatal within 30 hours, whereas no mouse in the 48-hour series died within 48 hours, and the first death in the 72-hour series occurred at 10 days. Against a total mortality of 100 p.c. in the 24-hour series and of 70 p.c. in the 48-hour series, only 20 p.c. died in the 72-hour series. It should be noted that serologically there is no difference between these three cultures. Sera prepared in rabbits from 24-, 48-, and 72-hour vaccines failed to distinguish between the three suspensions in terms of agglutinin titre and hence it may be assumed that they are all still in phase I.

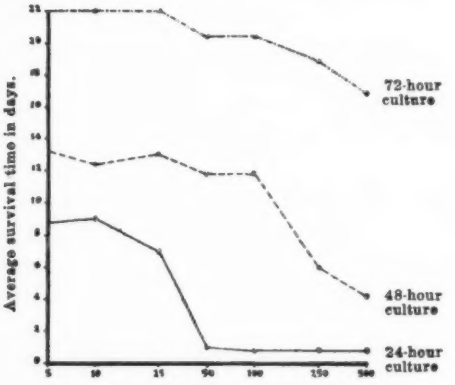

Fig. 7. Influence of culture age on the virulence of *H. pertussis* for mice based on average survival times.

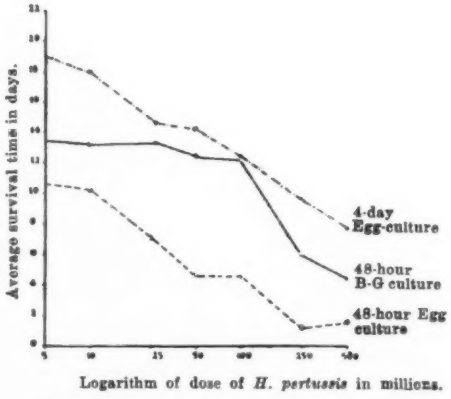
Average survival time in days.
4-day Egg-culture
48-hour B-G culture
48-hour Egg culture
Logarithm of dose of H. pertussis in millions.

Fig. 8. Comparative virulence of artificially cultured and egg-passaged *H. pertussis* for mice by the intranasal route.

Fig. 7 sets out the results graphically, plotting average survival times against the logarithm of the dose in millions. Analysis of these findings, using Fisher's exact χ^2 test, leaves no statistical doubt as to the significance of the observed differences between each comparable pair of the three series, whether the analysis be made in terms of percentage mortality or of average survival times.

The Influence of Culture Medium on the Virulence of H. pertussis.

Egg culture. Under the conditions of culture employed, which have been set out above, the minimum culture period in the egg yielding sufficient organisms for testing was 48 hours.

In the following experiment a 48-hour egg culture, a 4-day egg culture, and a 48-hour B-G culture, of which the virulence is already known, are compared. With experimental conditions as before, these cultures were prepared from a fresh ampoule, standardized and instilled into mice at the appropriate intervals in the manner described.

Table 2 summarizes this experiment, and in Fig. 8 the average survival times of the experimental groups are plotted against the logarithm of the dose of *H. pertussis* in millions.

The observed differences in virulence between the young and old egg cultures are statistically significant, whether assessed in terms of death or average survival times, and are in accordance with the findings relating virulence to physiological age, using B-G medium. There is no statistical doubt that the 48-hour egg culture was more virulent than the 48-hour B-G culture. How much this may have been influenced by errors in standardizing the egg suspensions (which operate to the disadvantage of the latter), can only be guessed, but it must be accepted on the basis of available figures that although the 48-hour egg culture is more virulent than the equivalent age on B-G medium, it is not more lethal than the 24-hour B-G culture. This is evidenced by comparing Figs. 7 and 8. It might reasonably be anticipated that a 24-hour egg culture would be more virulent than a B-G culture of the same age, but owing to technical difficulties mentioned earlier, this comparison was not made.

Further evidence of the enhanced virulence of egg cultures is afforded by the lack of significant difference between the 48-hour B-G culture and the 4-day egg culture, although the observed differences favour the former.

TABLE 2.

Comparative Mouse-Virulence of Artificially Cultured and Egg Cultured H. pertussis.

Series.	Inoculum.	No. of mice.	No. of organisms.	Individual survival time in days.	Survivors 21 days.	Avr. survival time.*
1.	Buffered broth	5	Nil	S,S,S,S,S	5/5	22 days
2.	10-day embryo fluid	5	Nil	S,S,S,S,S	5/5	22 days
3.	48-hr. egg culture	10	500 miln.	1,1,1,1,1,1,1,1,2,6	0/10	1·6 days
		10	250 miln.	1,1,1,1,1,1,1,2,2	0/10	1·2 days
		10	100 miln.	1,1,2,3,5,6,6,7,7,8	0/10	4·6 days
		10	50 miln.	2,2,2,2,2,3,7,8,9,9	0/10	4·6 days
		10	25 miln.	4,4,5,5,6,6,10,10,11,11	0/10	7·2 days
		10	10 miln.	7,9,9,9,10,10,11,12,12,13	0/10	10·2 days
		10	5 miln.	7,7,8,9,10,11,13,13,14,14	0/10	10·6 days
4.	48-hr. B-G culture	10	500 miln.	2,2,3,4,4,5,5,5,6,9	0/10	4·5 days
		10	250 miln.	2,3,4,5,6,6,8,8,9,9	0/10	6·0 days
		10	100 miln.	5,8,8,9,10,10,14,14,S,S	2/10	12·2 days
		10	50 miln.	5,8,9,9,10,12,13,16,S,S	2/10	12·4 days
		10	25 miln.	9,10,10,11,12,12,13,16,18,S	1/10	13·3 days
		10	10 miln.	5,8,8,9,10,12,16,S,S,S	3/10	13·2 days
		10	5 miln.	7,10,10,10,12,13,13,16,S,S	2/10	13·5 days
5.	4-day Egg Culture	5	500 miln.	2,5,7,9,16	0/5	7·8 days
		5	250 miln.	2,7,10,12,16	0/5	9·6 days
		5	100 miln.	10,11,11,14,16	0/5	12·4 days
		5	50 miln.	8,10,15,16,S	1/5	14·2 days
		5	25 miln.	9,16,16,16,16	0/5	14·6 days
		5	10 miln.	12,14,20,S,S	2/5	18·0 days
		5	5 miln.	14,15,S,S,S	3/5	19·0 days

* Average survival time. Mice surviving more than 21 days (S) are taken as having survived 22 days for the purpose of striking averages (see Fig. 8).

The Effect on Virulence of Serial Subculture on Bordet-Gengou Medium. The enhanced virulence of egg cultures over B-G cultures of the same age suggests that neither B-G medium nor any other artificial medium yet tested is optimal for the maintenance of *H. pertussis* in its most virulent form—a hypothetical state probably not reached under the conditions of these experiments. Leslie and Gardner (1931), Lawson (1939), and others, have previously produced evidence to show that fluctuations in agglutinability and virulence may occur under maintenance, without animal passage, on B-G medium, but others, such as North and Anderson (1943) and Toomey and his colleagues (1944) failed to detect any deterioration even after long periods in artificial culture.

To determine whether a relatively small number of generations on B-G medium reduces virulence, a comparison was made of a culture passaged for 24 generations at intervals of two to four days and an ampoule culture, both test suspensions being incubated 24 hours at 37° C. (Table 5).

TABLE 5.

Comparative virulence of dried and B-G cultured strains of H. pertussis.

Series.	Inoculum.	No. of mice.	Dose.	Survival time in hours.	No. surviving.	Avr. survival time.
1.	24-hr.	5	500 miln.	20*,20,20,20,20	0/5	20 hrs.
	vacuum	5	250 miln.	20,20,20,20,20	0/5	20 hrs.
	dried	5	100 miln.	20,20,20,20,21	0/5	20·2 hrs.
	culture	5	50 miln.	20,20,21,24,24	0/5	21·8 hrs.
2.	24-hr.	5	500 miln.	20,20,20,25,72	0/5	30·4 hrs.
	B-G passaged	5	250 miln.	20,20,20,24,24	0/5	21·6 hrs.
	(24 sub-	5	100 miln.	24,25,27,27,120	0/5	45·2 hrs.
	cultures)	5	50 miln.	24,26,40,48,120	0/5	51·6 hrs.

* 20 hours. This figure is taken to indicate deaths occurring overnight, but not within 5 hours of inoculation. The differences in average survival time between series 1 and 2 are therefore probably greater than the figures indicate.

The observed differences between the survival times of the two series may be considered, in several different ways, by each of which statistical analysis using Fisher's exact χ^2 technique gives a significant result. The distinction, however, is very fine, and it is not impossible that successive transfers might fluctuate to this extent in both directions. If any importance can be attached to these results it lies in the strength they afford the theory that cultures used for virulence tests or for vaccine production are best maintained in the dried state. That full virulence may be restored to this B-G passage culture by one mouse passage and also by cultivation in the developing egg for one generation is demonstrated in Table 6. In this experiment *H. pertussis* recovered from the lung of a mouse inoculated with 24th B-G passage culture and organisms recovered from a 7-day egg sown with this culture, were compared with an ampoule culture.

TABLE 6.

Restoration of virulence to H. pertussis *by mouse and egg passage.*

Series.	Inoculum.	No. of mice.	Dose.	Survival time in hours.	Average survival time.
1.	Vacuum-dried	5	500 miln.	20,20,20.20,21	20·2 hrs.
	culture	5	250 miln.	20,20,20,20,20	20·0 hrs.
	(24 hrs. at	5	100 miln.	20,20,20,21,21	20·4 hrs.
	37° C.	5	50 miln.	20,20,20,20,24	20·8 hrs.
2.	24th B-G	5	500 miln.	20,20,20,20,26	20·3 hrs.
	passage ex	5	250 miln.	20,20,20,22,23	21·0 hrs.
	mouse lung (24	5	100 miln.	20,20,20,22,22	20·8 hrs.
	hrs. at 37° C.)	5	50 miln.	20,20,21,23,24	21·6 hrs.
3.	24th B-G	5	500 miln.	20,20,20,20,20	20·0 hrs.
	passage ex	5	250 miln.	20,20,20,20,28	21·6 hrs.
	egg (24 hrs.	5	100 miln.	20,20,20,20,24	20·8 hrs.
	at 37° C.)	5	50 miln.	20,20,20,20,22	20·4 hrs.

DISCUSSION.

One might seek an explanation for the difference in virulence exhibited by the same strain of *H. pertussis* grown in the developing egg and on B-G medium in the assumption of a preponderance of virulent over avirulent cells in a medium more suited to the development of the former. The dramatic superiority of physiologically young cultures, on both media, however, is less easily interpreted. From the rapidity of the deaths in mice and the severe haemorrhagic oedema of the lungs noted the natural assumption appeared to be that is was due to labile toxic products resembling, perhaps in this respect, the soluble haemolysin of haemolytic

streptococci, but as the organisms are harvested from solid medium into which the bulk of a soluble exotoxin would probably have diffused, this seemed a remote possibility. Moreover, the literature on the toxic qualities of *H. pertussis* (Bordet and Sleeswyk, 1910; Evans and Maitland, 1937; Evans, 1940; Wood, 1940; Roberts and Ospeck, 1942; Flosdorf and Kimball, 1940; Katsampes and his colleagues, 1942; and Anderson and North, 1943) does not encourage one to assume that the organism produces an exotoxin. On the other hand, there appears to be definite evidence of endotoxin production, which increases progressively with the physiological age of the culture. This endotoxin, containing heat stable and heat labile fractions, has been demonstrated in fluid medium and in the washings of heavy suspensions of 48-hour B-G cultured organisms and was found to be produced in highest concentration by an avirulent phase III strain (Roberts and Ospeck, 1942). It is capable of stimulating antitoxin production only when extracted from the organisms and formalinized and not while in the intact cell, thus suggesting its intracellular situation. The antitoxin is stated to neutralize not only pertussis endotoxin, but also that of *H. parapertussis* and *Brucella bronchiseptica*, indicating that the endotoxin is nonspecific. Katsampes and his colleagues (1942) found it to be lethal for three-weeks-old mice on intravenous injection of a dose of 0·2 ml. of the washings of a 20,000 million 48 hour B-G suspension. Anderson and North (1943) found that death in mice following intraperitoneal inoculation with *H. pertussis* phase I could be prevented by antitoxic serum, but not by antibacterial serum, whereas with intranasal infection the reverse was true. Evans (1944) confirmed the inability of antitoxin to influence the course of intranasal infection and concluded that serum containing antitoxin and no antibacterial bodies is able to protect mice against infection with *H. pertussis*, only when there is sufficient antitoxin present at the site of the infection to neutralize the toxin in the infecting dose. This theory is not convincing. It might be suggested, from experience here, that death following massive intraperitoneal injections of *H. pertussis* is in fact predominantly of endotoxic origin. The virulence of young cultures on the other hand is associated with infectivity of the individual cell which is not related to endotoxin content.

Toxin Experiments.

To avoid the action of endotoxin (Katsampes and his colleagues, 1942) and to detect exotoxin, if it were present in young cultures, rabbits and three-weeks-old mice were inoculated with the centrifuge supernatant of suspensions containing 10,000 million organisms of 24-hour, 48-hour and 72-hour cultures, and of heavily sown 18-hour B-G buffered broth cultures. In doses ranging from 0·25 to 1·0 ml. in mice and of 0·5 to 5·0 ml. in rabbits, given intravenously, no evidence of toxic activity was obtained. Thus it would appear that neither endotoxin nor exotoxin may be held responsible for the enhanced virulence of young cultures. Moreover, the possible influence of capsules in this regard was ruled out by demonstrating, with the aid of an India ink method in which a carbol fuchsin counterstain is used, that there is no obvious diminution in capsules to be seen in serial smears taken at intervals of 7, 12, 24, 48, 72 and 120 hours on B-G medium.

The plate-counting technique of Miles and Misra (1938) failed to disclose a disparity between the number of viable cells in the three suspensions used sufficient to account for the observed differences in virulence. Furthermore, the smaller size of the organisms in a 72-hour culture suggests that the suspensions of the older cultures would contain greater total numbers per ml. in a given opacity, thereby loading the experiment against the younger culture.

One is then left with the conclusion that we have in the present instance no satisfactory theoretical basis upon which to assess the relative importance of cellular and extra-cellular components of *H. pertussis* in determining its virulence. Therefore, until this difficulty is overcome it appears necessary to adopt the purely empirical assumption that some of the properties conferring virulence are likely to be lost unless experimental conditions are very carefully controlled. It seems evident, however, that provided *H. pertussis* cultures are maintained

either in the dried state or, failing this, on B-G medium with frequent mouse lung passage, and provided physiologically young cultures are used, the intranasal route offers a reliable and rational method of testing the virulence of living organisms or the protection afforded by killed organisms. There appears to be little to recommend the continued use of the intraperitoneal route for either purpose.

The observations recorded here and those to follow have been made with the twofold objective of arriving at a reliable and generally applicable method of assessing vaccine potency, and of seeking an explanation for the divergent results following the use of various vaccine preparations. Experimental results now being prepared for publication have shown that virulence and immunizing potency are closely related, so that 24-hour, 48-hour and 72-hour vaccines confer respectively on mice a probability of survival of 86 p.c., 65 p.c. and 40 p.c. It is appropriate to mention here that whereas most American workers claiming successful protection of children used a 48-hour vaccine, McFarlan and his colleagues (1945) used a 72-hour vaccine.

SUMMARY.

Hitherto unsuspected variations in the mouse virulence of *H. pertussis*, within the prescribed limits of phase I of Leslie and Gardner, have been demonstrated. The important factor concerned is the physiological age of the culture, but minor variations may be induced by cultivation in the developing chick embryo or by serial sub-culture on Bordet-Gengou medium without mouse passage.

Enhanced virulence does not appear to be influenced by the amount of endotoxin present; in addition neither exotoxins nor capsules are concerned. It appears to be due to an increase in the infectivity of the individual cell, which is difficult to interpret in our present state of knowledge.

Under the conditions laid down, the intranasal method of infecting mice offers a reliable and rational means of determining both the virulence and the immunizing potency of *H. pertussis*.

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ACCOMMODATION OF NERVE IN MYASTHENIA GRAVIS AND IN PARTIAL CURARIZATION

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When an electromotive force is applied to a nerve for "long" periods, stimulation occurs only when the E.M.F. is at or above a certain level called the threshold value for the nerve. The excitation occurs only at make and break, not during the passage of the current. The threshold level for excitation at make is not constant, but varies proportionately to a number of factors. The factor concerned here is the rate at which the applied E.M.F. rises; the more slowly it rises the higher the threshold voltage. A.V. Hill (1936) considered this phenomenon and on theoretical grounds arrived at the conclusion that it might be due to an activity of the nerve acting in reverse direction to the excitatory changes produced by the applied current, and at a rate exponentially related to the excitatory effect already produced by the exciting current. He developed an equation relating the variation in threshold to the rate of rise of E.M.F. and the resultant factor λ, is an expression of the rate at which the nerve opposes the excitatory effect. The factor λ should be constant for all rates of rise of E.M.F. The measurements necessary to arrive at the factor are the threshold for an instantaneously rising E.M.F., and for a number of pulses of different rates of exponential rise of the applied E.M.F. relative to time. Solandt (1936) carried out the necessary experiments and found that λ was constant over a wide range of rates of rise of applied voltage.

The possibility that the direct cause of the symptoms of myasthenia gravis is the presence in the blood of a curare-like substance was brought forward by Harvey and Lilienthal (1941) and raised the possibility that the curare-like substance was developed in the thymus gland of these cases. Further, Burman (1940) found that myasthenic cases were sensitive to curare, which indicated an additive or synergistic effect. Transmission from nerve to muscle is greatly impaired in both conditions. No information was, however, available as to whether transmission in nerve fibres showed any variation from the normal in either condition. It was considered that resistance to excitation would be the most likely factor of stimulation which would be affected. As the factor λ is a measurable expression of the speed with which a nerve opposes an exciting current, an investigation of this factor in these two conditions was undertaken.

METHOD AND RESULTS.

Myasthenic Cases.

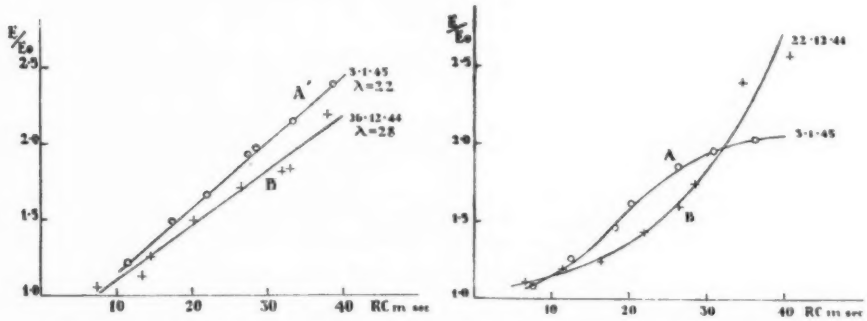
The method used in determining λ for human nerves was similar to that used by Solandt (1936). Currents increasing exponentially relative to time were produced by means of a fixed condenser of 10μF. capacity and a resistance variable up to 10,000 ohm. Different time constants of current rise were obtained by increasing the resistance by stages of 500 ohm from 500 to 4,000 ohm and then decreasing to 500 ohm in the same manner. At each stage of variation of the resistance by 500 ohm, the threshold voltages for the condenser in and out of the circuit, E_0 and E respectively, were measured by increasing the voltage from subthreshold to threshold strength. Excitation was indicated by tendon twitch felt at the wrist. The arm of the patient was first rubbed well with Zephiran (1/10) to reduce skin resistance. The stigmatic electrode, consisting of a thick zinc wire, wrapped in Zephiran-soaked cotton wool was placed over the ulnar nerve at the elbow, and kept in place with a rubber arm band. The indifferent electrode, a flat zinc plate covering a pad of Zephiran soaked cottonwool, was placed on the upper surface of the forearm. The measurements were usually taken as long as possible after the last dose of prostigmin, and then again 15–30 minutes after administration of prostigmin.

TABLE 1.

Values of λ obtained from determinations on cases of myasthenia gravis where linear relationships were obtained throughout.

Subject.	Date	Determination 2-3 hrs. after last dose of prostigmin.	Determination 30 mins. after prostigmin.	Dosage of prostigmin.
Mr. D.	8/5/45	34 m.sec.	30 m.sec.	(subcut.) 0·5 mg.
	10/5/45	62 "	40 "	" "
	18/5/45	28 "	28 "	" "
	12/6/45	40 "	38 "	" "
Mr. G.	13/6/45	25 "	18 "	(oral) 15 mg.
	13/6/45	28 "	62 "	" "
Mrs. M.	13/6/45	19 "	18 "	" "
Mr. L.	13/6/45	30 "	28 "	" "
Ian S.	13/6/45	11 "	19 "	" "
Miss D.	20/10/45	30 "	32 "	" "
	8/11/45	13 "	21 "	" "

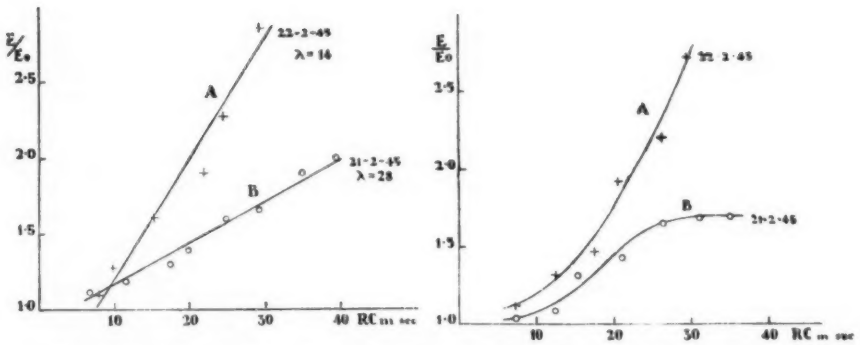
Eight probable cases of myasthenia gravis were studied, some more intensively than others. In five of the cases the curves were of the linear type predicted by Hill. The values of λ obtained are shown in Table 1. In three cases, however, a sigmoid or hyperbolic curve was usually obtained before the administration of prostigmin, while a linear relationship was always obtained from the determination made after prostigmin. In no case was there any noticeable change in threshold E_0 during the determination.



Figs. 1 and 1a. Mrs. V. Ordinate: E/E_0. Abscissa: m.sec. A and A′: Curves obtained on 3/1/45, 3 hours after last dose of prostigmin and 30 minutes after prostigmin (0·5 mg.) subcutaneously and 75 mg. by mouth respectively. B and B′ curves obtained on 26/12/44 at similar time relations to the same doses of prostigmin.

The curves shown in Fig. 1 and 1a were obtained for one of these cases (Mrs. V.). The symptoms were first evident four years ago, when the patient was 40 years of age. She maintained a fairly normal life on about 150 mg. prostigmin (taken orally) per day, until two months before this investigation was begun, when weakness became rapidly worse and she was admitted as an in-patient. During the period of the investigation she was receiving an injection of 0·5 mg. prostigmin subcutaneously and five 15 mg. tablets orally five times daily. The response to prostigmin was not marked, consisting of a slight improvement in her ability to move the fingers, and use the facial muscles. Definite enlargement of the thymus was shown radiologically. On all occasions λ was estimated three hours after the last administration of prostigmin and again 30 minutes after subcutaneous injection of 0·5 mg. and oral administration of 75 mg. of the drug.

The curves in Fig. 2 and 2a were obtained for Mr. G., aged 44, who having been an in-patient four years previously, at the onset of the disease, has for the past three and a half years maintained a fairly normal life at home on about 90 mg. of prostigmin daily, taken orally. No detectable enlargement of the thymus was shown radiologically.



Figs. 2 and 2a. Mr. G. Ordinate: E/E_0. Abscissa: m.sec. A and A′: Curves obtained on 22/2/45, 3 hours after last dose and 30 minutes after prostigmin (15 mg.) by mouth respectively. B and B′: Curves obtained on 21/2/45 at similar time relations to the same doses of prostigmin.

The curves in Fig. 3 were obtained from measurements made on another case, Miss O., aged 26. She had developed symptoms only recently and only one observation was made on her. The observations made before prostigmin was given do not fall on a straight line, but the curve drawn is probably not justified. There is, however, a contrast with that obtained after prostigmin. Since then she has reported that her condition has become much worse, speech being almost impossible unless prostigmin is taken, two tablets (30 mg.) enabling her to carry on normal speech for three hours.

TABLE 2.

		Preparation soaked in Curare Solution.				*Preparation soaked in Curare and Eserine Solution.*				
Date.	Time of Soaking (hrs.)	Tension p.c.	Type of Curve.	λ	Curare Concentration.	Tension p.c.	Type of Curve.	λ	Concentration. Curare.	Concentration. Eserine.
20/2/46	—		Linear	12	1:5,000		Linear	28	1:5,000	1:50,000
	5	30	Hyperbolic			25	Linear	40		
8/3/46	—		Linear	24	1:10,000		Linear	15	1:10,000	1:50,000
	2	90	Linear	15		175	Linear	19		
	4	50	Sigmoidal			175	Linear	70		
	6	5	Sigmoidal			No response.				
13/3/46	—		Linear	15	1:10,000		Linear	37	1:10,000	1:50,000
	2	150	Hyperbolic			150	Linear	23		
	4	100	Hyperbolic			100	Linear	21		
	6	100	Hyperbolic			10	Linear	8·5		
15/3/46	—		Linear	10	1:10,000		Linear	10·5	1:10,000	1:100,000
	2	100	Linear	9		100	Linear	9·5		
	4	10	Linear	4		100	Linear	8		
	6	6	Linear	10		80	Linear	13		
21/3/46	—		Linear	17	1:10,000		Linear	14	1:10,000	1:100,000
	2	110	Linear	8		120	Linear	7		
	4	80	Linear			120	Linear	12		
	6	4	Hypberolic			15	Linear	6		
25/3/46	—		Linear	4	1:10,000		Linear	6	1:10,000	1:100,000
	3	80	Linear	6		110	Linear	14		
	5	80	Linear	10		50	Linear	9		
26/3/46	—		Linear	7	1:5,000		Linear	9	1:5,000	1:100,000
	3	50	Hyperbolic			100	Linear	19		
	6	10	Hyperbolic			100	Linear	16		
1/4/46	—		Linear	9	1:5,000		Linear	14	1:5,000	1:100,000
	3	50	Linear	11		30	Linear	18		
	6	2	Hyperbolic			No response.				

Another case which was studied fairly intensively was that of Mr. D. He had severe symptoms of myasthenia gravis, but responded fairly well to prostigmin treatment (three injections (subcutaneous) of 0·5 mg. and three oral doses of 45 mg. prostigmin daily). There was no obvious enlargement of the thymus. The first symptoms had developed some six months prior to the investigation. In no instance was any deviation from the linear relationship observed. Results are shown in Table 1.

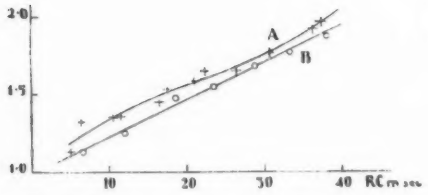


Fig. 3. Miss O. Ordinate: E/E_0. Abscissa: m.sec. A: Curve obtained before any prostigmin. B: Curve obtained 30 minutes after 15 mg. of prostigmin by mouth. See text.

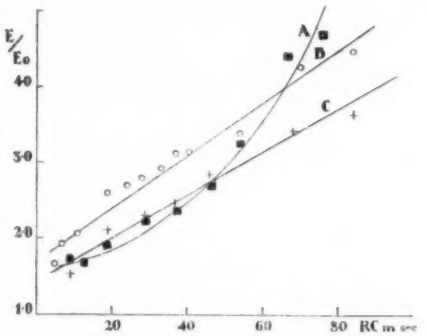


Fig. 4. Cat, sciatic nerve. Ordinate: E/E_0. Abscissa: m. sec. B: Curve obtained before giving "drugs". A: Curve obtained after intravenous injection of 2 c.c. decoction of crude curare. Response of muscle 50 p.c. of original. C: Curve obtained after intravenous injection of 2 mg. of eserine.

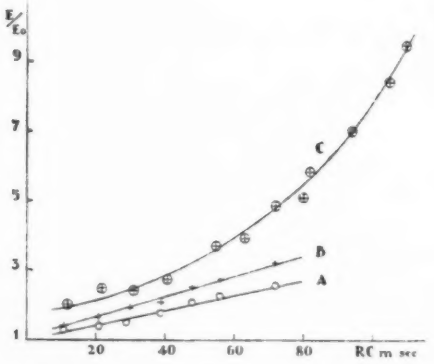


Fig. 5. Cat, sciatic nerve. Ordinate: E/E_0. Abscissa: m. sec. A: Curve obtained before administration of "drugs". $\lambda = 47$. B: Curve obtained after intravenous injection of 2 c.c. decoction of crude curare. Muscular response 95 p.c. of original. $\lambda = 32$. C: Curve obtained after intravenous injection of a further 2 c.c. decoction of crude curare. Muscular response 50 p.c. of original.

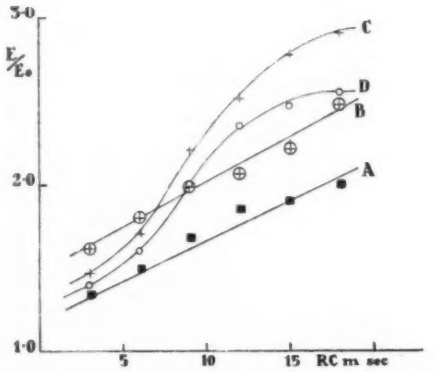


Fig. 6. Frog. Sciatic gastrocnemius preparation. Ordinate: E/E_0. Abscissa: m.sec. Soaking in 1:10,000 Intocostrin. A: Before application of "drugs". $\lambda = 22$. B: After soaking for 2 hours. Muscular response 90 p.c. of A. $\lambda = 18$. C: After soaking for 4 hours. Muscular response 50 p.c. of A. D: After soaking for 6 hours. Muscular response 5 p.c. of A.

Four other very mild cases of myasthenia were investigated. They also showed a linear relationship before and after prostigmin.

Curarized Nerve.

Mammalian. Using as little anaesthetic as possible, cats were decapitated and respiration maintained artificially. Insulated electrodes were placed on the distal part of the cut sciatic

nerve, and the tendo Achilles attached to an isometric lever recording on a smoked drum. The same circuit as for the human was used but the change of threshold with stages of 500 ohm was so small that stages of 1,000 ohm were used. The curare used was a crude decoction of curare given intravenously. The results obtained in the two cats are shown in Figs. 4 and 5. From these it can be seen that when the maximum response of the muscle is reduced to a slight extent only, the relationship is still linear, but when the condition is severe there is obtained a hyperbolic curve. Eserine re-establishes the linear relationship.

Frog. Sciatic gastrocnemius preparations were mounted in a muscle bath and the tendo Achilles attached to an isotonic lever recording on a smoked drum. The testing circuit in which a variable condenser and fixed resistance were used to vary the rate of rise of E.M.F. was of the same design as that used by Solandt (1936). Eight to ten settings of the condenser from 0·01–0·2μF were used and λ estimated for the nerve of the preparation.

The effect of curare on nerve accommodation was tested on eight pairs of nerve muscle preparations. One preparation was soaked in 1:5,000; or 1:10,000 Intocostrin (Squibb) in Ringer's solution, for periods of up to six hours; the other was similarly treated except for the addition of 1:100,000, or 1:50,000 eserine solution. Determinations were made before soaking and at various time intervals during the soaking. Records of the tension developed in a single twitch by the muscle using a maximal stimulus were taken at the same time.

Five preparations showed hyperbolic curves after soaking in curare, and one a sigmoidal curve. The curves obtained from preparations to which eserine had been added to the curare solution were in all cases linear. Two pairs of preparations gave linear curves throughout the period of investigation. These results are shown in Table 2. There was no clear relationship between the depression of the tension developed by a maximal shock, under the influence of the drug, and the appearance or non-appearance of linearity in the accommodation curves; however, soaking for 2–5 hours in curare solution was necessary before the non-linearity appeared. Fig. 6 is a typical curve from a preparation soaked in 1:10,000 Intocostrin in Ringer's.

DISCUSSION.

The results show that the nerve fibres may show a change in accommodation of a similar type in both myasthenia gravis and curare poisoning.

There appears to be little doubt that acetyl choline is involved in transmission across the neuro-muscular junction, and that curare prevents this action. Much evidence has recently been adduced that acetyl choline may play an important part in the transmission in some nerve fibres (Fulton, 1945; Feldberg, 1945). The parallelism which has previously been shown between the changes in neuro-muscular transmission in curare poisoning and in myasthenia gravis, must now be correlated with the parallelism between the changes in accommodation of nerve fibres in the two conditions. In the first place, it strengthens the evidence for a curare-like result in myasthenia gravis. In the second, it strengthens the inference that the mechanisms at the neuro-muscular junctions and in motor nerve fibres, (sensory nerves were not used here) have some mechanism, or mechanisms, sensitive to the same pharmacological substances. A further conclusion is that this mechanism is reflected in the dimension λ, which is an index of the rate of breakdown of the physico-chemical state leading to excitation. The opposition which eserine and prostigmin show to this action indicates the possibility that choline esterase is involved.

The hyperbolic nature of the curves obtained from myasthenia gravis patients and curarized nerve, indicates that these nerves resist excitation to an increasingly greater degree, as the time constant of current rise is lengthened. This may be due to an extra factor appearing in the complex of events which enables a motor nerve fibre to accommodate to a stimulus of long duration, or to the enhancement of an existing factor. The concave portion of the sigmoid curves may also be due to this same factor, whereas the flattening of the latter portion of the sigmoid curve is similar to the break in accommodation curves obtained by Rosenblueth and del Pozo (1942), from mammalian motor nerves (unsevered peroneal) when the time constant of current rise was about 150 m. sec. They ascribe this break to the existence of two different thresholds of excitation with different time constants of accommodation. In curarized nerve and in nerves from myasthenics, this break apparently appears when the time constant of current rise of the stimulating circuit is comparatively small.

SUMMARY.

In myasthenia gravis and curare poisoning of advanced degree, the $E/E_0/RC$, or accommodation curve of motor nerve fibres departs from a linear relationship, becoming sigmoid or hyperbolic.

This returns to normal when eserine or prostigmin is applied.

The relevance of these findings to the rôle of an acetyl choline mechanism in motor nerve fibres is discussed.

Acknowledgments: The "Intocostrin" used in the experiments was kindly given by Messrs. Squibb & Sons, New York.

Technical assistance was provided from a grant from the National Health and Medical Research Council.

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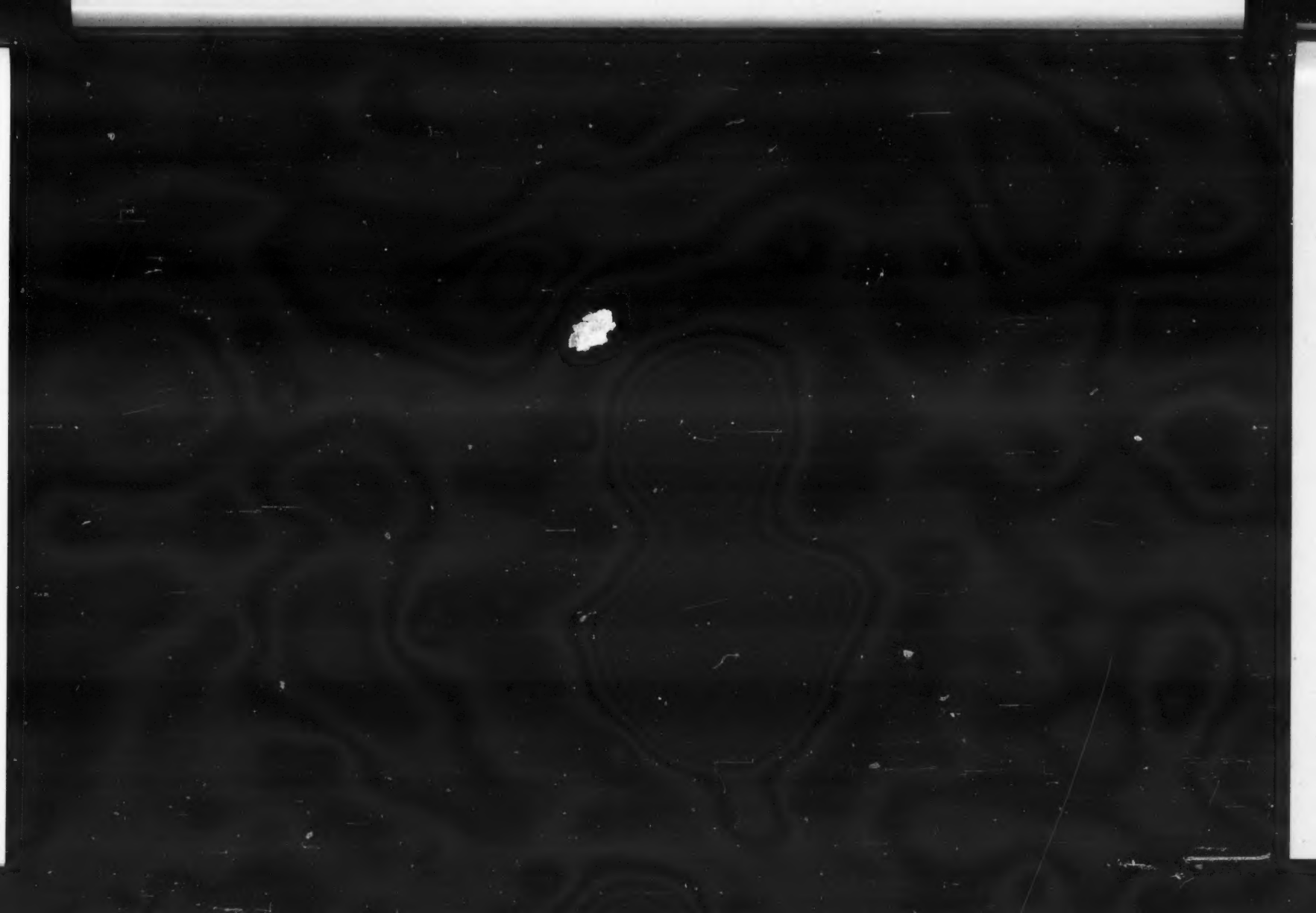

SIMPLE TECHNIQUES FOR THE PREPARATION OF MOULD MOUNTS

by V. B. D. SKERMAN

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(Accepted for publication, 1st August, 1946.)

The preparation of mould mounts for microscopical examination is frequently difficult. Textbooks on mycology offer little advice on the subject apart from the usual technique of removing a portion of the mycelium from the colony with a pair of stiff mounted-needles. The technique usually results in disarrangement and fragmentation of the mycelium and fruiting bodies which are not always desirable, and difficulty is experienced in freeing the material from the adherent substrate. For this reason it seemed desirable to find some procedure which would tend to obviate these defects in mounting technique. The following methods are recommended as satisfactory alternatives for many types of moulds.

Procedure: Cultivate the mould on a suitable medium, viz. Czapek-Dox in the usual manner. Discharge a drop of a mixture of 75 parts of ether and 25 parts of alcohol, from a drop bottle, over the area from which the preparation is to be taken. Allow this to evaporate slightly, but not completely, and then using a 4-millimetre loop, place a drop of the following solution within the moistened area:

Ether, 75 ml.
Ethyl alcohol, 25 ml.
Celloidin, 10 gm.

The celloidin will dry in one to three minutes as a thin film in which the mycelium is embedded. Remove the film with a pair of forceps as soon as it becomes sufficiently firm and transfer it to a slide.

In the majority of instances in which the mycelium is principally aerial the film can be removed without any of the agar adhering to it. In cases where the agar does adhere it can be removed by means of a scalpel without injuring the embedded structures. Subsequent treatment of the film depends on the density of the mycelium and the purpose for which the preparation is required. In all cases it is important to treat the films before they become too dry; otherwise great difficulty will be experienced in removing the celloidin.

For the examination of the arrangement of various structures as they occur in the culture it is essential to prepare the film from an area where the mycelium is not too dense. Pretreatment with the ether-alcohol mixture prevents the inclusion of air bubbles in the celloidin film and such preparations can be stained and examined without removing the celloidin.

Transfer the film to a clean slide and mount under a coverslip in lactophenol blue. This preparation may be examined after a few minutes without further treatment, or the film may be washed with water after a few minutes in the lactophenol blue and then mounted in clear lactophenol.

The success of this treatment depends on the density of the mycelium.

For routine examination of moulds where the mycelium is too dense for the previous treatment the celloidin is removed from the mycelium and the latter teased out prior to staining. Dissolve out the celloidin by treating the film with the mixture of 75 parts of ether and 25 parts of alcohol, preferably on a hot plate with a surface temperature of 45–50° C. Thin out the mycelium and remove excess spores by teasing the material with a suitable instrument and washing with the solvent. Mount the preparation in lactophenol blue or lactophenol yellow.

For preparations required for photographic purposes: Stained films mounted in clear lactophenol may yield preparations suitable for photographic purposes, especially if the film is stained with lactophenol blue previously diluted with twice its volume of clear lactophenol. The presence of the coloured mounting fluid used in the second method of treatment is undesirable and attempts to replace it with the clear mounting fluid are not very successful. The following method is therefore recommended for preparations required for photographic purposes.

Stain the mycelium by immersing the film in lactophenol blue for ten minutes. Remove and wash the excess stain off with water. Then immerse the film in warm (*ca.* 45° C.) alcohol until the stain no longer runs freely from it. (This removes most of the stain from the embedding medium.) Transfer the film to a clean slide and remove the celloidin with the ether-alcohol solvent. Tease and mount in clear lactophenol.

Fig. 1.

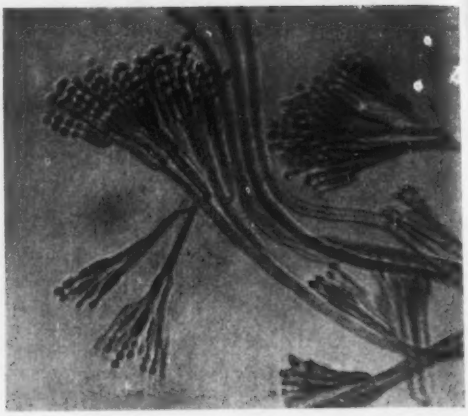

Fig. 2.

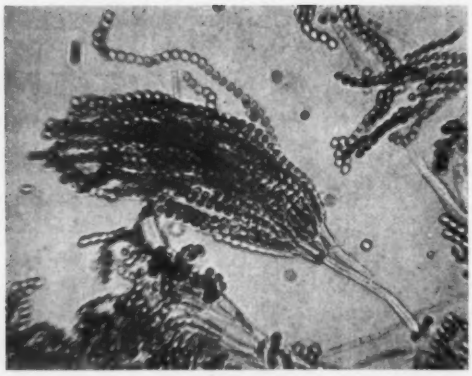

Fig. 3.

The accompanying photomicrographs are of preparations prepared by this method. Figs. 1 and 2 are of preparations which were teased and washed to remove excess spores. Fig. 3 shows an asymmetric penicillus with its long catenulate spore chains.

Permanent preparations may be prepared from any of the above by ringing the preparations with shellac as recommended by Smith (1946).

SUMMARY.

A simple technique is described for preparation of mould mounts for routine examination and for photographic purposes.

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SULPHUR DISTRIBUTIONS, WITH SPECIAL REFERENCE TO THE CYSTINE PLUS CYSTEINE AND METHIONINE CONTENTS, OF THE SEEDS AND SEED PROTEINS OF TWO VARIETIES OF SOYA BEANS

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(Accepted for publication, 29th August, 1946.)

There is considerable evidence (see Lugg, 1945a) in support of the suggestion that the seeds and seed "whole" proteins of any legume should be deemed of low methionine content unless actual experimental evidence exists to the contrary. The generalization has a wide validity, but until recently the available information has suggested (see Lugg, 1945b) that the seeds of the soya bean, *Glycine soja*, are rather exceptional. This seed has formed a staple article of human diet in Oriental countries for many centuries. Furthermore, although sundry feeding trials (e.g. Hayward and Hafner, 1941; and Almquist, Mecchi, Kratzer and Grau, 1942), which have been somewhat complicated by the presence of protease inhibitor in unheated material (see Ham and Sandstedt, 1944), have indicated that the methionine content was low enough to constitute a first limiting factor for the growth of the chick and the rat, they have not indicated a notable deficiency of this amino acid. Indeed, Almquist, Mecchi, Kratzer and Grau (1942) themselves, using McCarthy and Sullivan's (1941) method, estimated the methionine contents of raw soya bean "protein" and of soya bean meal "protein" to be 1·9 and 2·1 p.c. respectively. In making their calculations they appear to have multiplied the nitrogen in the materials by a conventional factor (presumably 6·25) and called the result "protein". On the assumption that they did this, their values may be recalculated to yield, respectively, 1·1 and 1·2 p.c., on a nitrogen basis (i.e. as percentages of the nitrogen represented by the amino acids concerned).[2]

Numerous estimates have been made of the contents of the sulphur-containing amino acids, cystine and methionine, in soya bean seeds and their mixed proteins, and there is considerable discordance between the results of even the more serious studies. Csonka and Jones (1934) extracted defatted soya bean meal with dilute solutions of NaCl and estimated the cystine in the acid-hydrolysed extracts by Csonka's (1932) modification of Sullivan's (1929 a, b) method. They computed the cystine contents of defatted seeds of a number of varieties to range between 0·37 and 0·63 p.c. on a nitrogen basis. Hamilton and Nakamura (1940) extracted defatted soya beans with dilute solutions of NaOH, submitted the extracts to acid hydrolysis and estimated the cystine contents by Lugg's (1933a) method which is based on Sullivan's (1926) reaction. From the results, they computed the cystine contents of seeds of eleven varieties to range between 0·38 and 1·04 p.c. on a nitrogen basis. When recalculated on a nitrogen basis, Block and Bolling's (1943–4) values for the cystine and methionine contents (estimated by inadequately specified methods) of soya bean meal protein prepared from commercial meal, become 0·7 and 1·2 p.c., respectively. More recently, and without commenting on their previous analyses, Block and Bolling (1945) have reported distinctly different

[1] On visit from the University of Western Australia.

[2] It is unfortunate that some authors report amino acid contents of protein preparations as percentages by weight and neglect to state the nitrogen contents of the preparations, thereby making it difficult to establish a basis for comparison of their values with those obtained by other investigators. We prefer, as usual (Lugg, 1938, and subsequent articles), to assume no arbitrary nitrogen content for impure protein preparations and to report amino acid contents on a nitrogen basis. Chibnall (1945) too, recommends the adoption of this procedure.

values (0·95 and 0·94 p.c., respectively, recalculated on a nitrogen basis) when using an inadequately-defined colorimetric method based on the reduction of phospho-18-tungstic acid for estimating cystine, and McCarthy and Sullivan's (1941) method for estimating methionine. Grau and Almquist (1945), using McCarthy and Sullivan's (1941) method, have estimated that the methionine contents of samples of raw soya beans and of solvent process soya bean meal were 1·2 and 1·3 p.c. respectively (recalculated on a nitrogen basis).

Except for some of the values obtained by Grau and Almquist (1945), who claimed that acid and enzymic hydrolyses yielded substantially the same results, these various estimates have been based upon analyses of acid hydrolysates of what may be regarded as impure protein preparations. There is, therefore, (see Lugg, 1933b, 1938, 1939; Bailey, 1937), the likelihood that considerable fractions of these amino acids were lost to estimation in the first place, but there is also the possibility that deficiences in the subsequent colorimetric procedures might have compensated in some instances and to an unknown degree, for the loss. There is the possibility, furthermore, that Hamilton and Nakamura's (1940) results may have been vitiated by the action of the alkali upon the cystine in proteins (see, e.g. Horn, Jones and Ringel, 1942).

Evans (1945) avoided these shortcomings by using a modification of Lugg's (1938) method for the estimation of cyst(e)ine and methionine. It has been pointed out by Lugg (1938) that when this method (and, of course, its modifications, such as that of Callan and Toennies, 1941, and that of Evans, 1945) are applied directly to proteins without prior hydrolysis, the grave uncertainties incidental to the acid hydrolysis of impure preparations are exchanged for another set of uncertainties which are likely to be of much smaller consequence. But Evans (1945) applied the method only to soya bean oil meal, and not to protein preparations derived therefrom. For twelve samples of material he reported cystine contents ranging from 1·31 to 1·53 p.c. and methionine contents ranging from 0·76 to 0·94 p.c. (recalculated on a nitrogen basis). The method is of too low a specificity to warrant the leaving of serious analytical work with seeds at this stage, and in previous studies with seeds (Lugg and Weller, 1941, 1944; Lugg and Clowes, 1945) we have been disinclined to rely upon such values alone, and have, almost invariably, considered them only in relation to values obtained also with "whole" protein preparations derived from the seeds.

These considerations, together with the fact that soya beans are becoming increasingly important as an agricultural crop in many parts of the world, have justified a limited but more critical examination of their sulphur distributions, both in a general sense and with further attention to the question of varietal differences.

EXPERIMENTAL.

The Preparations.

The study was confined to seeds of the varieties *Dunfield* and *Lincoln* of *Glycine soja* grown at Armidale, New South Wales, and harvested ripe, on 30/3/46. The seeds were ground rapidly, to avoid much gain or loss of moisture, and finely enough to pass through a sieve with holes 1·0 mm. sq.

TABLE 1.

Preparation of "whole" protein samples from seeds.

Seed material	Fresh wt. taken gm.	P.c. moisture	Dry wt. gm.	P.c. N	Total N mg.	Coagulable N mg.	Protein N mg.	Dry wt. protein gm.	P.c. N in protein prep.
10 *Glycine soja* var. *Dunfield*	40·0	11·8	35·3	5.68	1990	1825	1818	19·4	9·39
11 *Glycine Soja* var. *Lincoln*	40·0	10·0	36·0	5·74	2065	1874	1865	18·9	9·88

From samples of the ground materials "whole" protein preparations were made by extracting non-coagulable and other non-protein nitrogenous substances and certain other substances as well, by the method described by Lugg and Weller (1944). In Table 1 are shown the moisture contents of the seeds, the amounts of total N, coagulable N and protein N in them, the percentages of N in the "whole" protein preparations, and other data concerning the preparations. In direct continuation of the numbers used to characterize the preparations described by Lugg and Weller (1941, 1944) and Lugg and Clowes (1945), the two varieties of soya bean seeds (*Dunfield* and *Lincoln*) have been given the numbers 10 and 11, respectively.

Methods of Estimation.

All nitrogen estimations were made by the modified Kjeldahl method, in which selenium (conveniently added as SeO_2) is used as a catalyst, and the digest in the H_2SO_4–Na_2SO_4

mixture is maintained at the boiling point for at least 8 hours after it has become virtually colourless.

The cyst(e)ine (i.e. cystine plus cysteine, calculated as cystine) and methionine contents of the protein preparations and the distribution of sulphur in the seeds were estimated by Lugg's (1938) "differential oxidation" method, the procedure described by Lugg and Weller (1944) being followed in the estimations of total S.

RESULTS.

In Table 2 are shown the cyst(e)ine and methionine contents of the "whole" proteins of the seeds as percentages of the nitrogen present occurring in these forms, and the sulphur distributions in the seeds are also shown as cyst(e)ine and methionine contents. The sulphate-S contents, too, have been expressed in relation to the nitrogen present. The fundamental assumptions in the application of the method are (1) that the sulphur is present only as cyst(e)ine and methionine (free, or as amino acid residues in proteins) and sulphate, and (2) that the amino acid residues behave with the reagents in the same ways as do the free amino acids. Reference to the validity of these assumptions has been made by Lugg (1938, 1939) and by Lugg and Weller (1944), and the subject need not be discussed in detail here. It is held that from available evidence the assumptions appear to be reasonably justified for protein preparations even when these are heavily contaminated with impurities of a carbohydrate or polyuronide nature. But, as various seeds are known to contain other compounds of sulphur, part (1) of the assumptions cannot be expected to hold for unextracted seeds. Hence, in accordance with the practice of Lugg and Weller (1944) and Lugg and Clowes (1945), we have shown the cyst(e)ine and methionine contents of the seeds in italics to indicate the distinct possibility of the values having been vitiated by the inclusion of other compounds of sulphur. Replicate estimations of the sulphur fractions were made and the values shown were calculated from the means. The random errors of the values shown, assuming that their deviations from the true means are unlikely to exceed twice the "standard error", are believed not to exceed 0·04 for cyst(e)ine, 0·05 for methionine, and 0·05, 0·02, 0·02 and 0·01 for sulphate in 10, 10W, 11 and 11W, respectively.

TABLE 2.

Partial compositions of the preparations: losses during extraction.

No.*	Preparation from	P.c. N	P.c. Cyst. N	P.c. Meth. N	$\frac{SO_4\text{-}S}{N} \times 100$	P.c. loss, or apparent loss, during extraction. Cyst.	Meth.	SO_4-S
10	*Glycine soja* var. *Dunfield* (seeds)	5·68	*1·48*	*0·93*	0·12	37	14	70
10W	*Glycine soja* var. *Dunfield* (seeds)	9·39	1·01	0·88	0·04			
11	*Glycine soja* var. *Lincoln* (seeds)	5·74	*1·70*	*0·92*	0·05	44	17	80
11W	*Glycine soja* var. *Lincoln* (seeds)	9·88	1·06	0·85	0·01			

* The suffix "W" signifies the "whole" protein preparation made from material bearing the characteristic number.

DISCUSSION.

As Table 2 shows, there was a pronounced similarity in the sulphur distributions of the two varieties of seeds, and an even closer similarity in the cyst(e)ine and methionine contents of the "whole" protein preparations derived from them. Indeed, to within the random errors of estimation the "whole" proteins must be considered to have possessed the same cyst(e)ine and methionine contents.

Apparent losses of cyst(e)ine and methionine and losses of sulphate during the extractions in the preparation of 10W from 10 and of 11W from 11, are shown on the right of Table 2. The qualification "apparent" is necessitated by the possibility, which has been mentioned earlier, of the cyst(e)ine and methionine values for 10 and 11 having been vitiated by inclusion of other sulphur-containing compounds. Whereas the random errors of estimation might conceivably account for

much, though perhaps not all, of the methionine losses, it is considered that a different explanation must be sought for the bulk of the cyst(e)ine losses. The appropriate view, in the light of the earlier remarks, is that the unextracted seeds could have contained very little methionine other than that combined as amino acid residues in protein, but that approximately 30 p.c. of the apparent cyst(e)ine in the seeds was non-protein cyst(e)ine or was, perhaps, represented by sulphur compounds other than cyst(e)ine.

Of the various legume seeds we have examined (Lugg and Weller, 1941, 1944; Lugg and Clowes, 1945; and the present study), these soya bean seeds lie at the one extreme in that their "whole" proteins have proved, in terms of the nitrogen present, to be of the highest methionine content. The corresponding cyst(e)ine contents are lower than those of the "whole" proteins of embryos from the ripe and unripe seeds of the several species of *Lupinus*, but are greater than those of the "whole" proteins of ripe and unripe seeds of the variety of *Pisum sativum* and of the "whole" proteins of ripe seeds of the variety of *Trifolium subterraneum*.

It is an interesting fact that our values for the methionine contents of the "whole" proteins of the two varieties are smaller than any of those we have mentioned in the introductory remarks as having been obtained by other investigators by the application of a colorimetric method to acid hydrolysates of the proteins. On the basis of the claimed high specificity of the colour reaction and the presumptive losses during acid hydrolysis, the reverse might have been expected. Our values for the cyst(e)ine contents, on the other hand, are greater than most of those obtained by the application of colorimetric methods to the hydrolysates.

Our values for the apparent cyst(e)ine and methionine contents of the unextracted seeds are similar to those obtained by Evans (1945) for soya bean oil meal, but most of the methionine values he reported were smaller than those we obtained.

Evans' (1945) modification of the method was tested by him with free cystine and methionine, but not with a protein in which the sulphur distribution had already been well established by other means. There is, therefore, more uncertainty about its ability to distinguish sharply between cyst(e)ine and methionine residues than there is with Lugg's (1938) original method. However, it is a very minor modification, employing approximately 15 N instead of approximately 22 N HNO_3 in one phase of the procedure, and it might thus be expected to behave more nearly like the original method than perhaps could be expected of the modification of Callan and Toennies (1941), in which alkaline permanganate is used in place of the nitric acid.

At all events we feel that the conclusion must be reached that, in terms of the nitrogen present, the seeds and the seed "whole" proteins of the soya bean are of rather low methionine content, and that even if all the apparent non-protein cyst(e)ine in the seeds is cyst(e)ine in the free state or otherwise combined, the total cyst(e)ine content is only moderately high. It seems, moreover, that the influence of seed variety upon the contents of these amino acids in the seed "whole" proteins is probably slight, but that the sulphur distributions in the unextracted seeds may be rather more strongly affected.

In the light of what is known of the dietary methionine requirements of animals like the rat, the chick and man, and the sparing action of cyst(e)ine (see e.g. Rose, 1937; Grau and Almquist, 1943; Rose, Haines and Johnson, 1942; Madden, Carter, Kattus, Miller and Whipple, 1943), the soya bean seeds and their "whole" proteins cannot be regarded as of high nutritive value, though they are perhaps superior in this respect to any other legume seed materials we have examined and markedly superior to some of them.

SUMMARY.

The seeds of two varieties of soya bean (*Glycine soja* var. *Dunfield* and *G. soja* var. *Lincoln*) have been examined with regard to the sulphur distributions and with particular reference to the cyst(e)ine and methionine contents of the "whole" proteins.

The cyst(e)ine and methionine contents of the "whole" proteins were very similar for both varieties, but in a general sense the variation in the sulphur distributions in the unextracted seeds was more pronounced.

Only small amounts of methionine, other than that combined as amino acid residues in protein, could have been present in the seeds, but rather more of the cyst(e)ine could have been present, in the free state or in other combinations.

In terms of the nitrogen present, the seeds and "whole" proteins were of moderate cyst(e)ine content and of rather low methionine content. They are therefore, not to be regarded as deviating seriously from the general pattern of legume seeds discussed earlier (see Lugg, 1945a).

Brief reference has been given to the implication of these findings in the sphere of animal nutrition.

Acknowledgments. We are indebted to Mr. A. H. Williams, of the Commonwealth Department of Commerce and Agriculture, and to the New South Wales State Department of Agriculture for the supply of seeds, and to Mr. A. M. Gallacher for assistance. One of us (R.J.) wishes to thank Professors V. M. Trikojus, E. J. Underwood and N. S. Bayliss for arrangements, and the Institute of Agriculture, University of Western Australia, for travelling expenses.

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THE PHARMACOLOGICAL INTER-RELATIONS OF THE MUSCLE STIMULATING SUBSTANCE IN SERUM[1]

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Although it has been known for at least sixty years that defibrinated blood possesses vasoconstrictor properties, the pharmacological and chemical nature of the substance responsible has excited surprisingly little interest. It has been named "vasotonin" or "vasoconstrictine" (Amberson, 1937) and "spatgift" by the Germans. Recently, the name "thrombocytin" was suggested (Reid, 1943) because it arises from blood platelets, a fact known to the earlier observers and confirmed most recently by Zucker (1944).

When serum is used for the assay of other vasoconstrictor agents which may be present in blood, the presence of this substance has been commonly forgotten. Even when plasma is used for such purposes, thromocytin may be present in small, and occasionally in large quantity, due to breakdown of platelets during preparation (Reid and Bick, 1942; Landis *et al.*, 1943). In recent years there has been considerable interest in vasoconstrictor agents in the circulating blood in experimental and clinical hypertension, in shock, and following haemorrhage (Page, 1943; Dexter *et al.* 1943; Gregory *et al.* 1945, and many others). It is important, therefore, to investigate the properties of the substance (or substances) liberated during clotting or by platelet break-down. Reports on its pharmacological relationships have yielded little positive information. The main facts about its chemical nature have been reported elsewhere (Reid and Bick, 1942; Zucker, 1944).

In an earlier paper the origin of substances in human serum which contract the guinea-pig intestine, the ox carotid artery, and the vessels of the perfused rabbit's ear was studied. At that time we were not concerned with the extent to which histamine, which is reported in serum (Barsoum and Gaddum, 1935; Code, 1937, 1937a; Code and Ing, 1937), may have been responsible. Histamine constricts the vessels of the rabbit's ear and not uncommonly causes contraction of the arterial strip. So far as the intestine is concerned, the latency of several seconds is against the response being due to histamine; but it is possible that the presence of other substances may have affected the latency. Although serum also contracted the uterus of the virgin rat, which is relaxed by histamine, part at least of this activity was nonspecific, *i.e.* not due to substances formed during clotting.

This paper presents evidence that the vaso-constrictor and muscle-stimulating activity of serum and platelet extracts is not attributable to histamine. It also reports observations on the pharmacological inter-relations of the substance responsible.

METHODS.

Sera from man, dog, cat, ox and guinea-pig were prepared by allowing whole blood to clot, the sera being separated by centrifugation. With the exception of the guinea-pig specimens, which were used immediately, the sera were collected with aseptic precautions, or sterilized by filtration after separation. Protein-free filtrates were prepared by precipitating the protein with trichloracetic acid and extracting the filtrate with ether to remove the acid. Some activity was lost in this process, which is a modification of the method of Barsoum and Gaddum (1935) for the estimation of histamine in blood. In their method the filtrate is boiled with

[1]Technical assistance provided by a grant from the National Health and Medical Research Council.

acid for an hour or longer, a procedure which they show destroys "spatgift"; in these experiments it was unboiled. Filtrates were also prepared by adding an equal volume of normal saline to a sample of serum, bringing to the boil, cooling, adding N/1 hydrochloric acid to precipitate the protein, filtering and neutralizing the filtrate. Platelet extracts were made as described previously.

The isolated jejunum of the guinea-pig, spiral strips of carotid artery of dog or ox and spiral strips of dog jugular vein were used as test-objects. The preparations were mounted at 37° C. in isolated organ baths of 12 c.c. volume. Injections were made intravenously into cats under chloralose anaesthesia and the blood pressure in the carotid artery recorded by Hg. manometer. Histamine quantities are expressed in terms of the acid phosphate.

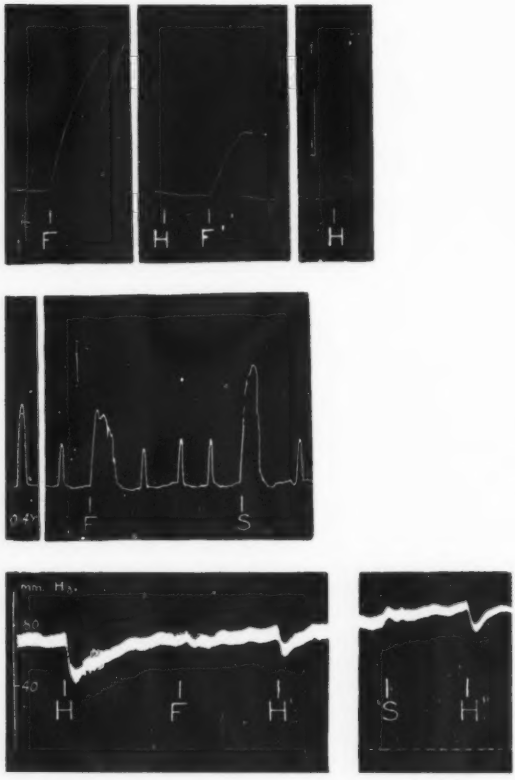

Fig. 1. Effects of histamine and a filtrate of ox serum on the ox carotid artery (upper panel) jejunum of guinea-pig (middle panel), and blood pressure in a carotid artery of a cat under chloralose anaesthesia (lower panel). See text for details.

RESULTS.

TO WHAT EXTENT IS THE ACTIVITY DUE TO HISTAMINE?

The action of histamine solutions was compared with that of serum, filtrates, or platelet extracts:

On Different Kinds of Preparation.

Twenty-nine samples of serum, serum filtrates or platelet extracts were tested on the jejunum of the guinea-pig, on the arterial strip and by injection into the cat under chloralose anaesthesia. One such experiment is illustrated in Fig. 1. The upper figure shows the response of the ox carotid artery to 2 c.c. and 1 c.c. doses of a filtrate of ox serum at F and F′ respectively, and of 1μg. of histamine at H, the second injection giving a small response. Clearly, if the activity of this filtrate were due to histamine, 1 c.c. must contain considerably more than 1μg. The middle figure shows the response of the jejunum to 0·5 c.c. of the same filtrate at F and 0·2 c.c. of the corresponding serum at S. The unlettered contractions are due to 0·2μg. of histamine. Again, if all the activity were due to histamine the filtrate could not contain more than 0·8μg. per c.c. Tested on the blood pressure of the cat (see lower figure) no histamine could be detected in 7·5 c.c. of filtrate at F, or 5 c.c. of serum at S. Doses of histamine of 1·0μg., 0·5μg., and 0·2μg. were injected at H, H′ and H″ respectively.

Generally speaking, it was not possible to compare the actions of histamine and serum on the blood pressure of the cat, as the serum, due to its protein content, commonly caused a "Brodie effect". To overcome this difficulty in sensitive animals, protein-free filtrates were used, or the cat was desensitized by repeated serum injections in the course of the experiment.

Such experiments showed clearly that histamine could not be the main substance causing muscular contraction when sera or filtrates from ox, dog, cat or man were used. Guinea-pig serum was richer in histamine, but here it represented only a fraction of the activity. Thus, in Fig. 2, 0·2 c.c. of guinea-pig serum at S gave an immediate response somewhat less in size than that due to 0·1μg. of histamine, followed by a large response after a latency of nine seconds. Generally speaking, tested on the blood pressure of the cat, filtrates of guinea-pig serum con-

tained histamine equivalent to 0·4 to 1·0μg. per c.c. of serum. These amounts approximated those calculated from the immediate responses of the jejunum of the guinea-pig. These results are in agreement with those of Barsoum and Gaddum and of Code, whose results indicate that the histamine content of guinea-pig blood which was of the order of 1μg. per c.c., is some 10 to 30 times that of the blood of man, dog or bullock, and over 100 times that of cat's blood.

At Different Times Using the Same Type of Preparation.

Tested on different occasions on the same type of preparation or even the very same preparation, the activity of serum or filtrates relative to that of histamine was widely variable. Some preparations were more sensitive to histamine than to serum, while others showed the reverse relationship. It will be shown later how prolonged contact of the intestine with serum reduced or abolished the contraction initially set up by the serum, while potentiating the action of histamine.

Before and After Treatment of the Test-Object with Thymoxyethyldiethylamine.

Thymoxyethyldiethylamine when added to the isolated organ bath in a dose of 5μg. depressed the response of the gut to potassium and to acetyl choline as well as to histamine. It is not, as would appear also from the records of others, (Climenko *et al.*, 1941; Rosenthal and Brown, 1940; Rosenthal and Minard, 1939) a satisfactory specific antagonist to histamine. The response to both histamine and serum is markedly depressed by this dose. However, during the period of recovery, occasionally, the response to histamine is greatly depressed, while serum or filtrate gives a good response. This is illustrated in Fig. 2, in which the effect of 0·2 and 0·1 c.c. of guinea-pig serum is seen at S and S′ respectively, the arrows indicating the extent of the immediate responses. Between the two main portions of the figure, 5μg. of thymoxyethyldiethylamine were added. The response to histamine is depressed considerably more than that to serum, 0·1 c.c. which previously gave a response less than that of 0·4μg. of histamine now has a greater effect than 1·0μg.

Thymoxyethyldiethylamine in doses of 5 to 10μg. was without effect on the response of the arterial or venous strips to serum or filtrates.

Before and After Incubation with Histaminase (Torantil).

Experiments in which sera or filtrates were incubated with histaminase, showed very clearly that the muscle-stimulating substance was not destroyed by such incubation, while control solutions of histamine in saline or serum lost activity. Serum was incubated at 37° C. for three to four hours, during which time there was a loss of some activity; but the activity of serum and serum containing one unit of Torantil per 4 c.c. (a large dose) remained equal. One unit in 20 c.c. of serum or saline containing 2μg. of histamine per c.c. destroyed all the histamine present.

It was considered possible that the mechanical breakdown of cells in the preparation of platelet extracts may have liberated large amounts of histamine not present in serum, and led in the past to false conclusions about the activity of such extracts. However, it was shown that the main activity of such extracts could not possibly have been due to histamine, for reasons similar to those above, and by the fact that such extracts caused contraction of arterial preparations on which histamine is without action. Thus, Fig. 3 shows the response of the carotid artery of the dog to 0·5 and 0·25 c.c. of an extract of dog platelets at E and E′ respectively. At H and H′ 3μg. and 10μg. of histamine were given.

PHARMACOLOGICAL INTER-RELATIONS.

1. *Influence of Atropine and Eserine.*

(a) *On the arterial strip.* Atropine in dosage from 1 to 250μg. is without effect on the response of the arterial strip of ox or dog to the vasoconstrictor substance in serum. This is so whether it is added during a contraction or some minutes beforehand. Eserine also is without effect on the response. The lower portion of Fig. 4 illustrates these points. The increase in size of the response to each successive injection of 0·5 c.c. of ox serum at the marks is a usual feature. The response of the ox carotid strip is unaffected by 2·0μg. of atropine sulphate between the first two panels and 1·0μg. of eserine salicylate at E.

(b) *On the isolated gut.* Not uncommonly atropine reduced the response to histamine and serum as well as to acetyl choline, but with suitable dosage a differential effect could readily be observed. This is illustrated in the upper portion of Fig. 4. At the arrows are the responses to 0·1 c.c. of guinea-pig serum. At the strokes 0·5μg. of acetyl choline was added. The unmarked contractions are to 0·1μg. of histamine. Between the first two panels 0·2μg. of atropine was given. The specific reduction in the response to acetyl choline and its recovery on washing out the bath can be seen, the effect on histamine or serum responses being only slight.

Eserine caused strong contraction of the gut and no information could be obtained on its effect on the response to serum.

Fig. 2.

Fig. 2. Effects of histamine and guinea-pig serum on the isolated jejunum of the guinea-pig. Between the fourth and fifth panels 5μg. of thymoxyethyldiethylamine were added to the bath and left in contact for 5 minutes before washing out. Other details in text.

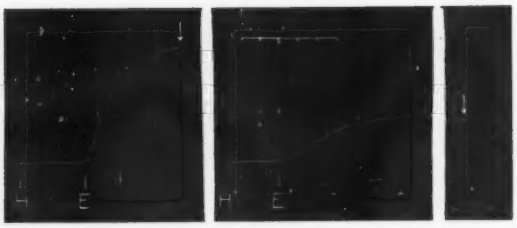

Fig. 3.

Fig. 3. Effects of histamine (10μg. at H') and an extract of dog platelets (at E and E') on the carotid artery of the dog. At the arrows the bath was washed out. Interval between panels: 5 and 10 minutes respectively. Time in minutes. Details in text.

Fig. 4. Upper figure: responses of guinea-pig jejunum to 0·4μg. of acetyl choline at strokes, to 0·1μg. of histamine (unlettered contractions) and to 0·2 c.c. of guinea-pig serum (at arrows). Between the first two panels 2·0μg. of atropin were left in contact for 5 minutes.

Lower figure: responses of ox carotid artery to ox serum. For details see text.

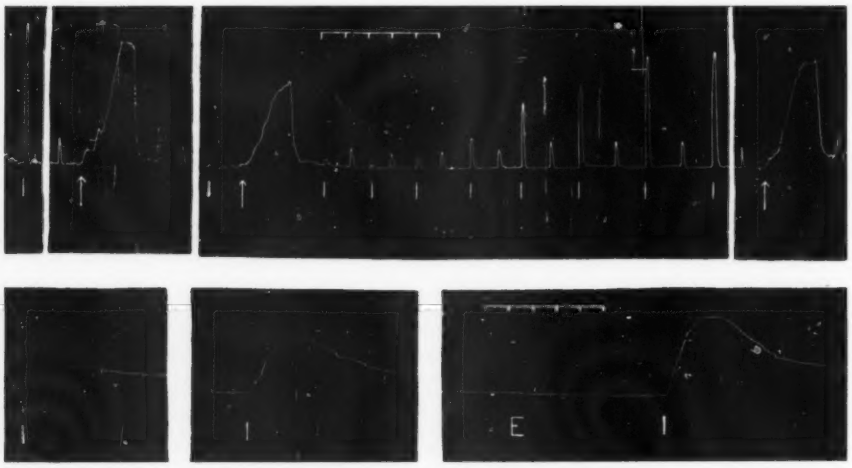

Fig. 4.

2. *Influence of Ergotamine and Yohimbine.*

(a) *On the vascular strip.* When ergotamine tartrate in doses of from 50 to 500μg. was added to the bath during a sustained contraction, no effect or a slight reduction in contraction was observed. When added to the bath before the second addition of serum it caused a gradual contraction which made subsequent results difficult to interpret, further serum injections caus-

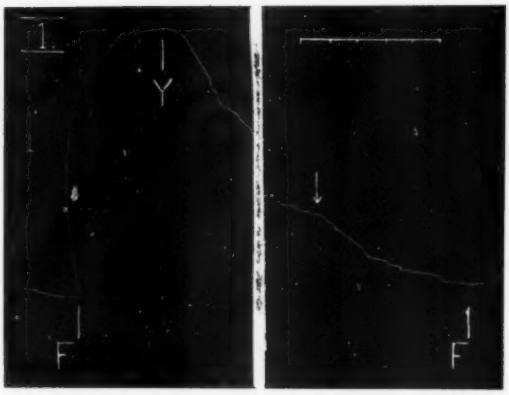

Fig. 5. Response of ox carotid artery to a filtrate of ox serum at F. At Y 100μg. of yohimbine was added to bath which was washed out at arrow. Interval between panels was 3 minutes. Time in minutes.

ing only rarely a reduced response —*i.e.* the total contraction due to both ergotamine and the second addition of serum was rarely less than that due to the first serum injection. Yohimbine did not cause contraction of the strips and was more regular in its effects, reduction in the response to serum or filtrates being commonly observed. This was more effective when added before an injection of serum than during a sustained contraction. In six experiments when added during the height of contraction a dose of 100 to 250μg. had no effect in two cases, a trivial effect in two, and a marked effect in the other two. In no instance, however, was the lever returned to the baseline until the bath was washed out. When yohimbine was added before serum it caused pronounced reduction or abolition of response in three of four experiments on the ox artery, and had no effect in one experiment with the jugular vein of the dog. Fig. 5 illustrates the response of the ox carotid artery to 2 c.c. of a trichloracetic acid filtrate of ox serum at F. At Y 100μg. of yohimbine hydrochloride reduced the response. Washing the bath out at the arrow caused further relaxation. The interval between the panels was three minutes. A second injection of filtrate at F′ produced no effect. Twenty minutes later after repeated washing, the full response to filtrate had returned.

(b) *On the gut.* Adrenaline in doses of from 0·5 to 1·0μg. relaxed the jejunum. This also took place if the gut were already contracted in response to serum. Yohimbine in doses of 125 to 250μg. reduced the effect of adrenaline or caused it to give a positive response. In similar dosage yohimbine caused partial relaxation of the gut contracting in response to a serum injection. However, yohimbine also relaxed the gut during a spontaneous change of tone, so that its effect cannot be regarded as specific. Similar results were obtained when ergotamine tartrate in doses of 40μg. was added to the bath during a sustained contraction, but relaxation was less complete than with yohimbine and took place more slowly.

When yohimbine in doses of 125 to 250μg. or ergotamine tartrate in doses of 40 to 125μg. was added to the bath before a serum injection, the subsequent response to serum was reduced or abolished, but not to a greater degree than the response to histamine. From these doses full recovery of response occurred, but at no stage of recovery was there an action specifically on the serum response.

3. *Influence of Cocain.*

Because of the relationship between sympatholytic agents on the response of arterial strips and perfusion preparations to the vasoconstricting action of serum, the effect of cocain hydrochloride was investigated. No evidence of potentiation by cocain in doses of 5 to 75μg. could be demonstrated on the isolated artery. It was common for the response both to serum and adrenaline to be increased after adding cocain to the bath, but such preparations usually increase in sensitivity with repeated injections of serum or adrenaline alone, a fact which makes interpretation difficult.

4. *Adrenaline and Calcium Ions.*

As stated above, adrenaline caused relaxation of the gut. Following an injection the response to both histamine and to serum was impaired for periods up to about 5 minutes. Calcium chloride added to the bath in amounts from 1 to 10 mg. had a like effect. Yohimbine abolished or reversed the response to both adrenaline and calcium ions, this effect outlasting its action on the response to serum or to histamine. At such a stage adrenaline or calcium ions no longer relaxed the gut during a serum contraction.

On the arterial strip sometimes adrenaline in doses of 1 to 10μg. caused relaxation; sometimes similar or larger doses were without action or caused contraction. When relaxation was produced serum given before or within two minutes of washing out the bath caused a

reduced response. Calcium in doses up to 50 mg. had usually no effect on the response of the carotid artery.

5. *Other Drugs.*

On the vascular strips sodium nitrite in doses of 10 to 50 mg. relaxed the preparation. While the drug remained in contact the addition of serum or filtrate caused no effect or a diminution in the expected response. This antagonism was more marked when the nitrite was administered before the serum than during a sustained contraction due to serum. It was not specific.

Histamine (up to 10μg.) and acetyl choline (up to 50μg.) when added during a sustained contraction were without effect on the response of the artery to serum, filtrates, or platelet extracts.

The addition of magnesium ions to magnesium-free Tyrode reduced the response of the gut to serum; it also reduced the spontaneous rhythm and the response to histamine. Magnesium ions were without significant action on the arterial preparations.

EFFECT OF PROLONGED CONTACT OF SERUM ON PREPARATION.

In an earlier paper it was stated that when the bath is washed out, some 30 seconds after the addition of serum, sometimes the response of the jejunum to histamine doses is at first decreased and later increased before returning to the original level of sensitivity. In the experiments recorded here, the effect of more prolonged contact of sera or filtrates on both the gut and the arterial strip was investigated.

On the Gut.

When serum or filtrate is left in contact with the gut for periods longer than one to three minutes, the contraction is not sustained but falls away, to or close to the baseline. This effect is not due to damage to the muscle, because the gut gives, while the serum is still in contact, a good (often potentiated) response to histamine, and may show spontaneous contractions. It is not due to destruction of active substances in the bath, because the contraction is not sustained even when serum is added to the Tyrode solution in the reservoir and the bath washed through every few seconds. Likewise, in one experiment a Tyrode-serum mixture taken from one bath after the gut had relaxed, when added to another preparation caused a contraction. For the same reasons the failure to maintain a response cannot be due to the accumulation of a metabolite in the bath. If it is due to some other substance in serum then it must have similar properties to thrombocytin, viz. can be obtained in protein-free filtrates and will withstand brief boiling. Without a pure sample this cannot be determined. Another explanation may be that serum stimulates the gut (possibly through the nerve tissue present) giving a phasic contraction, the set-up remaining refractory until the serum is removed. It still responds to increased concentrations of the serum. (See Fig. 6).

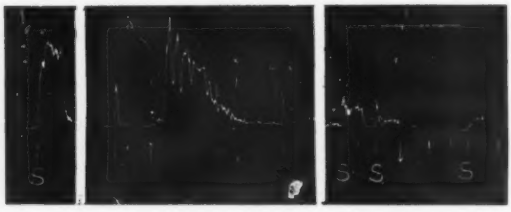

Fig. 6. Effect of prolonged contact of guinea-pig jejunum with ox serum (between arrows). Time in minutes, interval between 2nd and 3rd panels was 5 minutes. Details in text.

Fig. 6 shows the response of the jejunum of the guinea-pig to 2·0 c.c. of ox serum at S, the serum being left in contact for 90 seconds. At the strokes 0·1μg. of histamine was given. At the first arrow, the Tyrode solution in the reservoir was changed for a Tyrode-serum mixture of approximately the same proportion as that found in the bath after the first injection S, and the mixture run through into the bath, washing out every half-minute or so. A large contraction with rhythmic contractions resulted, the lever returning to the baseline in a few minutes. The gut still gave a good response to histamine at the strokes and also responded to an increase in serum concentration when 2·0 c.c. of serum were added for 90 seconds at S. At the second arrow Tyrode was reintroduced into the system, following which it was 13 minutes before the original sensitivity to histamine had returned.

On the Arterial Strip.

Serum, filtrate, or platelet extract, left in contact with the arterial or venous strip for periods up to one hour caused a sustained contraction followed by relaxation when the bath was washed out. (See Fig. 3).

Effect of Serum on the Response to Histamine and Acetylcholine.

Fig. 6 shows how the response to histamine was impaired after prolonged contact with serum but unimpaired while serum remained in contact with the gut. This observation was repeatedly confirmed; indeed, with contact of two minutes or longer histamine or acetyl choline added before washing out the bath caused usually potentiated responses. Fig. 7 illustrates these facts. Both the upper and the lower left-hand panels show the record of one piece of jejunum and the lower right-hand panel the record of a piece from another pig. At S 2·0 c.c. of ox serum were added to the bath, and 0·1μg. of histamine at the strokes. This gave a potentiated response when given before the serum was washed out at the arrow and after washing out a greatly reduced response. That the potentiation is not due to the fact that the bath was not washed out for some minutes is shown in the second panel, where between the second and third histamine responses the Tyrode was unchanged. In the lower left-hand panel a similar effect is seen following an injection at F of 2 c.c. of a filtrate prepared by boiling. In the lower right-hand panel the response of a piece of jejunum to 2 c.c. of a trichloracetic acid filtrate of ox serum is seen at F'. Here the filtrate was left in contact for two minutes, the potentiation but not the depression being obtained. The unlettered responses are to 0·2μg. of histamine. These experiments indicate that the substance responsible for the histamine potentiation, if not the muscle stimulating substance itself, is associated with it in protein-free filtrates. Filtrates of inactive plasma did not show this effect, although inactive plasma itself did so, indicating that some of the potentiating action of serum may be due to the protein or other nonspecific substance present.

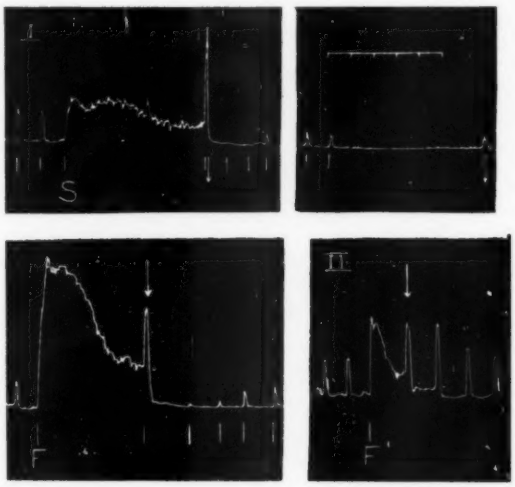

Fig. 7. Effect of serum and serum filtrates on the response of guinea-pig jejunum to histamine. Time in minutes. Details in text.

DISCUSSION.

The German observers (Guttentag, 1931; Zipf and Hulsmeyer, 1933) showed that "spatgift" was not histamine. Later on, Barsoum and Gaddum (1935) and Code (1937) measured the histamine content of blood and serum. Barsoum and Gaddum illustrate by one of their figures that other muscle-stimulating substances are destroyed in their histamine extraction; but the question remains as to what degree histamine contributes quantitatively to the activity of serum or platelet extracts.

We have in effect approached the problem by a method of difference in determining causal relations. Lacking a specific antagonist to histamine we have removed the possibility of its action in serum by incubating serum with histaminase without significantly affecting the activity. We also find, on comparing the effects of sera, filtrates or platelet extracts on the one hand with histamine on the other, that there is non-concomitance:

(a) From one kind of preparation to another.
(b) From one occasion to another on the same preparation.
(c) From one synergic or antagonistic reaction to another.

It is clear from these results that little or none of the muscle-stimulating activity of sera or platelet extracts of ox, dog, cat, guinea-pig, or man is due to

histamine. This is in agreement with the observations of Zucker (1944) who found that the response to serum of the nictitating membrane of the cat is not abolished when serum is incubated with histaminase or by injecting atropine.

It may, however, be mistaken for histamine when tests of serum or blood products are made only on the isolated gut of the guinea-pig. Since this substance may be produced in the absence of clotting, by platelet breakdown, its presence may seriously interfere with estimations of histamine in blood acted on by various tissue-injuring agents. Ungar (1944), for example, appears to have overlooked this when he used the guinea-pig jejunum to assay the histamine liberated by the action of peptone on blood without extraction of the histamine. The fact that he considers the procedure justified because the results agreed with those obtained when the histamine was extracted by a standard procedure is possibly attributable to the fact that he used rabbit blood, which is very rich in histamine. These present observations emphasize that in order to carry out histamine estimations in blood it is necessary to extract the histamine by a method such as that of Barsoum and Gaddum (1935), and to confirm the estimation by the use of preparations other than the guinea-pig intestine.

Recent interest in vasopressor substances in blood, also gives interest to this substance. As Landis *et al.* (1943), point out, it is essential in the estimation of such pressor substances to ensure (by "pre-heparinization" of the animal) that platelet breakdown does not occur in obtaining samples. In such studies knowledge of the pharmacological relations of thrombocytin would be helpful.

Unfortunately these observations have given few positive results. The observations of Heymanns *et al.* (1932), Bayliss and Ogden (1933) and Bing (1941) that ergotoxine abolishes its action in perfusion preparations led to an investigation of the action of other sympatholytic agents on the response of isolated vascular strips. Both yohimbine and ergotamine were found to abolish or reduce its action, but the effect was variable (as, indeed, it is with adrenaline antagonism). Stewart and Harvey (1912) found that the action of serum on the perfused kidney was unaffected by apocodein—another sympatholytic agent. The action of cocain on the response of the arterial strip was difficult to interpret, because such preparations usually increase in sensitivity. At best potentiation was doubtful. Zucker (1944) states that the response of the nictitating membrane to serum was increased by cocain.

It is interesting that whereas prolonged contact of serum on the gut does not cause a sustained contraction, contraction of the artery or vein is well sustained. This would be in keeping with the possible physiological role of thrombocytin—contraction of local parts of the vascular system when blood is shed or platelets disintegrate.

SUMMARY.

In sera of the ox, dog, cat, guinea-pig and man the main substance stimulating the guinea-pig jejunum or the arterial strip of ox or dog is not histamine.

Its action on the guinea-pig intestine or on the isolated arterial strip is reduced or abolished by yohimbine and less regularly with ergotamine.

Neither cocain, atropine, nor eserine affect the response of the arterial strip to sera or platelet extracts.

Thymoxyethyldiethylamine does not affect the response of the artery but considerably reduces the response of the intestine to serum.

Adrenaline and calcium ions relax the intestine and also reduce its response to serum. Sodium nitrite, histamine and acetyl choline when they relax the artery, also reduce its response to serum.

When serum or protein free-filtrates of serum are left in contact with the

isolated intestine the contraction ceases in a few minutes, but the responses to acetyl choline or histamine are potentiated until the bath is washed out; then they are depressed. Prolonged contact with the arterial strip causes a sustained contraction.

The importance of taking account of this substance when histamine or vasopressor substances in blood are estimated, is emphasized.

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